Effect of Indomethacin on Matrix Metalloproteinases in the Rat Kidney: Does Zinc Affect These Changes?

DISSERTATION

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In

BIOCHEMISTRY- BRANCH XIII

By

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September 6, 2007.

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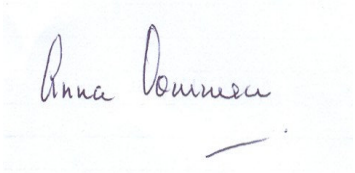
Dear Sir,

This is to certify that the study "Effect of Indomethacin on Metalloproteinases in Rat Kidney" by Dr Gautham T.P. was approved by the Institutional Animal Ethics Committee in consultation with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (IAEC No 2/2006).

Thanking you.

Yours sincerely,



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Department of Biochemistry, 20th September, 2007

Christian Medical College,

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This is to certify that this study entitled "***Effect of Indomethacin on Matrix Metalloproteinases in the Rat Kidney: Does Zinc Affect These Changes***?" is the bonafide work of **Dr. Gautham T.P.**, who carried out the work in the Department of Biochemistry under my guidance and supervision. The work in this dissertation has not been submitted to any other university for the award of a degree.

Dr. Molly Jacob, M.D., Ph.D.
Professor and Head,
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Department of Biochemistry, 20th September, 2007

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INTRODUCTION

The ability to relieve pain has been one of the major achievements of modern medicine. However in spite of advances in this science the cornerstone of pain control still remains opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Opioids were one of the oldest and most efficacious ways for man to alleviate the sufferings of pain and remain so even today. Issues of dependence and toxicity have marred the reputation of opioids and have defined strict protocols for their access and use by trained health professionals in specific clinical scenarios. The history of NSAIDs begins with the therapeutic use of willow bark to cure fever, aches and pains and date back to the time of Hippocrates (460 B.C.- 377 B.C.), only to be later clearly documented by Rev. Edmund Stone in 1763. Similar actions were seen with potions from meadowsweet (*Spiraea ulmaria*). Kolbe synthesized salicylic acid in 1853, and by 1874 it was being produced industrially. In an attempt to modify the toxicity profile of salicylic acid, Felix Hoffmann, a chemist with Bayer, in 1899 initiated animal testing of acetylsalicylic acid which later proceeded into human testing and marketing as aspirin, the first among the many NSAIDs to come.

NON-STEROIDAL ANTI INFLAMMATORY DRUGS

Nonsteroidal anti-inflammatory drugs are a group of chemically dissimilar molecules, which have similar analgesic, antipyretic and antiinflammatory activities and share similar toxicity profiles. NSAIDs exhibit their therapeutic effects by inhibiting the isoforms of cyclooxygenase, resulting in a net decrease in prostaglandin synthesis.

These drugs are often clinically classified as traditional NSAIDs (tNSAIDs) or COX-2 selective NSAIDs based on their specificity for inhibition of the various isoforms of cyclooxygenase. On the basis of chemical structure tNSAIDs are further classified as salicylates (aspirin, diflunisal), para-amino phenol derivatives (acetaminophen), acetic acid derivatives (indomethacin, sulinidac

and etodolac), fenamates (mefenamic, meclofenamic, and flufenamic acid), hetroaryl acetic acid derivatives (tolmetin, ketorolac and diclofenec), propionc acid derivatives (ibuprofen, narpraxon, ketoprofen), oxicams (piroxicam, meloxicam), nabumethone and pyrazolon derivatives (phenylbutazone, oxyphenbutazone). Attempts to minimize the toxicity of tNSAIDs, which are non-selective for COX isoforms, lead to the development of the COX-2 selective inhibitors "the coxibs" (Valdecoxib, celecoxib). The optimism of victory over adverse events of tNSAIDs was however short lived as studies with COX-2 selective inhibitors demonstrated an increase in mortality due to cardiac adverse events. The balance between the suffering of pain and adverse effects of treatment with NSAIDs still remains a clinical reality and a challenging field in research.

Current clinical status of NSAIDs

NSAIDs are among the largest group of drugs sold worldwide. Statistics from the National Prescription Audit, 1999-2000, indicate 111,400,000 NSAID prescriptions in United States for the year ending in August 2000, which amounted to a net value of approximately $4,800,000,000. The sales of over-the-counter analgesics (60% of which were NSAIDs) added an additional 3 billion dollars to this amount (Laine, 2001). There are reports that suggest prevalence of once-weekly NSAID use among people in the U.S. Among those who are 65 years old or older, usage may be as high as 70% with about half this group taking at least 7 doses per week (Talley et al., 1995). Data of similar nature is scarce in the Indian scenario, but it is safe to assume that similar patterns may exist. The market for NSAIDs in India was estimated to be approximately Rs1034.00 crores annually with a growth rate of 6.4% per year. These figures are possibly crude indicators of NSAID use in India. Hospital based studies indicate that approximately 65% of outpatient prescriptions contain NSAIDs (M. Guptha, 2005).

NSAIDs are often ideal drugs in clinical situations as their pain relieving ability is associated with a biochemical anti-inflammatory activity. The efficacy to these drugs has been established in dental pain (Ong and Seymour, 2003), osteoarthritis (Schnitzer, 2002), rheumatoid arthritis, ankylosing spondylitis (Zochling et al., 2006), gout, dysmenorrhea (Connolly, 2003) and headache (Lipton et al., 1998).

Other than being used as anti-inflammatory-analgesics, NSAIDs have other indications in therapy. Indomethacin, ibuprofen and other NSAIDs have been used to achieve total ductal closure in patients with patent ductus arteriosus (PDA) (Thomas et al., 2005). Aspirin and ketoprofen along with histamine receptor antagonists have been used to provide relief from episodes of vasodilatation and hypotension in patients with systemic mastocytosis (Worobec, 2000). Indomethacin, in combination with spironolactone and potassium repletion is used to improve the biochemical derangements and symptoms associated with Bartter's syndrome (Zawada, 1982). NSAIDs, such as aspirin and sulinidac, and more recently the coxibs have shown to have a cancer chemoprotective effect (Muir and Logan, 1999), wherein epidemiological studies have demonstrated decreases in the occurrence and progression of colon (Flossmann and Rothwell, 2007) and lung cancers (Harris et al., 2007). Aspirin is used, along with large doses of the antihyperlipidemic drug niacin, to reduce PGD_2 -mediated intense flushing, a major criterion limiting the tolerability of niacin (Oberwittler and Baccara-Dinet, 2006).

In recent years, COX-2 specific NSAIDs have made lauded as much-needed alternatives to conventional NSAIDs. However, recent reports on increased incidence of cardiovascular events among users of COX-2 inhibitors have dampened this enthusiasm. In this scenario, conventional NSAIDs continue to be of relevance today as they continue to be a necessity in the treatment of chronic arthritis and other related conditions.

Adverse effects of NSAIDs

The promise of pain relief by NSAIDs is often vitiated by toxicity, which most often present as gastrointestinal and/or renal complications. Extremes of age, pre-existing morbidities, and co-administered drugs, often increase the risk of developing adverse effects.

1. Gastrointestinal toxicity

The most common symptoms of toxicity associated with NSAIDs are gastrointestinal, and include anorexia, nausea, dyspepsia, abdominal pain and diarrhea. Clinically, they have been correlated with increases in the incidence of upper gastrointestinal ulcerations, bleeding, perforations and strictures (Abraham and Graham, 2005). NSAID induced gastric and intestinal ulcerations were among the first noted complications of this group of drugs (Langman, 1970). However it is now realized that in addition to these effects use of NSAIDs is associated with toxic effects in the small bowel, colon and rectum with alterations in intestinal function and permeability (Thiefin and Beaugerie, 2005).

2. NSAID-induced renal dysfunction

Approximately 1-5% of patients taking NSAIDs manifest a spectrum of nephrotoxic effects (Whelton and Hamilton, 1991). Even though the percentages are small, these figures translate into very large numbers considering the incidence and prevalence of NSAID use. NSAIDs are considered relatively safe in healthy individuals but the chances of developing renal complications increase to almost 20% in patients at high risk (Bush et al., 1991).

The risk factors for developing acute renal dysfunction following NSAID administration include age over 60 years, vascular disease, real or functional volume depletion, preexisting renal insufficiency, high renin-angiotensin states and systemic lupus erythematosus (Bush et al., 1991). Hyponatremia has been previously described in two groups of patients, infants treated with

indomethacin for patent ductus and adults and the elderly who have diseases or are taking drugs that impair urinary dilution (Rault, 1993). Elderly age, potassium spring diuretics, preexisting renal insufficiency predispose patients to develop hyperkalemia (Zimran et al., 1985, Hay et al., 2002). Acute interstitial nephritis has been observed at all ages and is difficult to predict (Bush et al., 1991). The development of analgesic nephropathy is associated with long term treatment with NSAIDs, analgesic abuse and certain drug-analgesic combinations used in treatment (Mackinnon et al., 2003).

Clinically, renal adverse effects caused by NSAID toxicity, manifest as follows in a decreasing order of frequency:

1. Fluid and electrolyte imbalance
    a. Sodium chloride and water retention
    b. Only water retention causing severe reversible hyponatremia
    c. Hyperkalemia
2. Acute deterioration of renal function (acute renal failure)
3. Nephritic syndrome with acute interstitial nephritis
4. Analgesic nephropathy with papillary necrosis

Most of the toxic effects of NSAIDs described, are often attributed to low prostaglandin levels as a consequence of COX inhibition (Ejaz et al., 2004). Fluid retention is the most common adverse effect of NSAIDs occurring in almost all patients who are exposed (Whelton and Hamilton, 1991). NSAIDs reduce renal blood flow and glomerular filtration rate (GFR) resulting in sodium retention and salt sensitivity (Morgan and Anderson, 2003). Water retention and toxicity as a result of NSAID use has been previously described in infants and the elderly (Petersson et al., 1987). These conditions often manifest clinically as edema (Carder and Weston, 2002), hyponatremia (Blum and Aviram, 1980) or increased blood pressure (Morgan and Anderson,

2003). However it is often possible to exercise caution and predict the occurrence of these complications in patients at risk (Bush et al., 1991).

NSAIDs have been shown to increase serum creatinine levels in patients with compromised renal function (Whelton et al., 1990). A similar study by Gurwitz and coworkers demonstrated that short-term use of NSAIDs caused reversible azotemia in a sizable proportion of elderly individuals (Gurwitz et al., 1990). The risk of developing renal dysfunction in the elderly increases with concurrent use of loop diuretics (Gurwitz et al., 1990) or angiotensin converting enzyme (ACE) inhibitors (Jolobe, 1999) which are often used to treat co-morbidities like hypertension. NSAIDs have been shown to increase the risk of developing acute renal failure (ARF) by 58% in the elderly (Griffin et al., 2000). Studies currently predict that the use of NSAIDs increases the risk of the first diagnosis of acute renal failure by 3-fold in the general population (Huerta et al., 2005).

Complications involving the renal parenchyma are more difficult to predict and are fortunately rare (Bush et al., 1991). Acute tubulointerstitial nephritis is a condition characterized by edema of the renal interstitium and interstitial infiltration by eosinophils, mononuclear cells and plasma cells (Rossert, 2001). The pathogenesis of tubulointerstitial nephritis is thought to involve direct cytotoxicity, inflammation and hypersensitivity (Rastegar and Kashgarian, 1998). NSAID induced interstitial nephritis is a reversible condition which may occur with or without nephritic syndrome (Ejaz et al., 2004) and has a reported prevalence of 1 in 5000 to 10,000 treated patients (Murray and Brater, 1993). Interstitial nephritis has been reported with the use of both tNSAIDs and COX-2 selective drugs (Szalat et al., 2004, Brewster and Perazella, 2004).

Classical analgesic nephropathy is associated with habitual consumption of predominantly analgesic products and may take several years to develop. The pathological hallmark of this

condition is interstitial fibrosis and the insidious development of renal failure (Henrich, 1998). Renal papillary necrosis is one of the first features to appear and gradually develops into progressive secondary cortical degeneration on further analgesic abuse (Gregg et al., 1989). This progresses into end stage renal failure with up to 20% of the patients needing dialysis or renal transplantation (Nanra et al., 1978). Chronic analgesic abuse is associated with the development of upper urothelial carcinomas as shown by animal studies (Hultengren et al., 1965) and observational studies in humans (Handa, 1979). However these results have not been established by case control studies (Linet et al., 1995).

INDOMETHACIN

Indomethacin is a methylated indole derivative, which like other NSAIDs has prominent antiinflammatory and analgesic-antipyretic properties. It is believed to exert its analgesic effects on both the central nervous system and locally at the site of inflammation.

Indomethacin is known to exert its antiinflammatory activity by inhibiting both isoforms of cyclooxygenase (Shen and Winter, 1977) in a concentration dependent manner (Kulmacz and Lands, 1985). The process of inhibition of COX is time-dependent and involves an initial competitive reversible phase followed by an irreversible phase characterized by a decay of enzyme activity, which is not be reversed by dilution (Smith and Lands, 1971). The resultant enzyme had a net activity of 4-10% of the original enzyme. It is postulated that the time-dependent effects are mediated due to conformational changes in the enzyme and do not involve any covalent modifications (Kulmacz and Lands, 1985).

As indomethacin is highly lipid soluble, it is well absorbed orally, achieving 80-100% bioavailability. Peak plasma concentration is achieved within 1 to 2 hours after a single oral dose. The drug is 90% bound to plasma proteins and has an association constant of $0.86\times10^{3}\ M^{-1}$

(M=molarity). Albumin is the predominant binding protein with an estimated 15 binding sites per molecule. Serum levels in patients with long term indomethacin therapy measured after a last dose of 25mg was found to range from 1.5-3μg/ml (Hvidberg et al., 1972). Indomethacin crosses the blood-brain barrier within 30 minutes of an intramuscular injection and achieves CSF concentrations similar to plasma concentrations within 2 hours. However analgesic activity was not found to correlate with neither serum nor CSF concentrations (Bannwarth et al., 1990).

The half-life of indomethacin is variable because of enterohepatic circulation but averages around 2 ½ hours. Studies with ^{14}C labeled indomethacin indicate that 50% of the drug administered by intravenous route underwent enterohepatic recirculation. Absorption and/or reabsorption of the drug happen to be more rapid and uniform by the rectal route (Kwan et al., 1976).

Indomethacin in its free form and as its glucoronide conjugate is found in early plasma samples after oral doses. These free and conjugated forms of indomethacin are excreted in bile, hydrolysed in the intestine and reabsorbed by enterohepatic circulation and are responsible for variations in half-life. O-demethylation and N-deacylation constitute other major metabolic pathways for the conversion of indomethacin into inactive metabolites and may occur independently or in a sequential manner (Duggan et al., 1972). Studies with ^{14}C labeled indomethacin to assess O-demethylation demonstrated the formation of O-desmethylindomethacin, N-deschlorobenzoylindomethacin and O-desmethyl-N-deschlorobenzoylindomethacin (Harman et al., 1964) as the major metabolites in this pathway. The formation of O-desmethylindomethacin is thought to be a major pathway in man as it represents 40-55% of the indomethacin excreted in the urine (Duggan et al., 1972). This conversion of indomethacin to its demethylated metabolite in humans, occurs in the liver microsomes, catalysed by P450 2C9 (Nakajima et al., 1998). The N-deacylation pathway is thought to occur independently of CYP P450 system and is catalyzed by a carboxylesterase

localized to the microsomes in pig liver (Terashima et al., 1996). This carboxylesterase catalyzed hydrolysis of indomethacin has also been noted in human and rabbit liver homogenates but is absent in rats and mice (Terashima et al., 1996).

Further metabolism of O-desmethyl N-deschlorobenzoylindomethacin (DMBI) has been noted in activated neutrophils by hypochlorous acid and the myeloperoxidase system to give rise to reactive iminoquinone intermediates (Ju and Uetrecht, 1998).

About 10-20% of indomethacin is excreted unchanged in urine, partly by tubular secretion. Free and conjugated metabolites are excreted through bile, urine and feces.

Indomethacin-induced renal dysfunction

The renal toxicity of indomethacin is well documented in literature both when used alone and in combination with other nephrotoxic agents. Indomethacin is known to cause disturbances in fluid and electrolyte balance, hypertension, acute renal failure, acute interstitial nephritis and chronic interstitial nephritis with renal papillary necrosis. There are also reports of renal dysgenesis (Restaino et al., 1991) and renal dysfunction (Buderus et al., 1993) following exposure to indomethacin in utero.

The most common renal adverse effect is sodium and water retention (Somova et al., 1984) and (Baker et al., 2005). Similar evidence has been noted in experimental animals also (Bartha and Hably, 1978). Indomethacin has also been noted to cause hyponatremia in infants and elderly patients with renal disease who have a preexisting impairment of urine concentrating mechanisms (Rault, 1993). Hyperkalemia in a background hyporeninemic hypoaldosteronism is an adverse effect which is more frequently associated with indomethacin than other NSAIDs (Tan et al.,

1979), (Goldszer et al., 1981) and (MacCarthy and Stokes, 1979). However the pathogenesis of hyperkalemia has been a subject of much debate as controlled experiments failed to demonstrate these effects conclusively (Padhani et al., 1987, Clive et al., 1992).

- *Acute renal failure due to the renal effects of indomethacin occurs in elderly (Gary et al., 1980) or hypovolumic patients (Weinberg et al., 1985). Acute interstitial nephritis is a reversible condition which has been described with the use of several NSAIDs, including indomethacin (Green et al., 1985). Long-term use of indomethacin with or without other analgesic agents is associated with the development of chronic irreversible interstitial nephritis and renal papillary necrosis (Jackson and Lawrence, 1978).*

- ***Other adverse effects:***

- *Indomethacin like other NSAIDs has been associated with other toxicities such as gastropathy (Filaretova et al., 2002), enteropathy (Zuccato et al., 1992) and hepatotoxicity (Kelsey and Scharyj, 1967). Therefore indomethacin serves as the prototype drug to study the effects of toxicity of traditional NSAIDs.*

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OXIDATIVE STRESS

Oxidative stress refers to a whole spectrum of changes in tissue or cellular function in response to a mismatch in the rate of formation of reactive oxygen species (ROS) or reactive nitrogen species and the rate of their removal by antioxidant defense mechanisms. Such changes in the redox status of the internal cellular environment play an important physiological role in anti-microbial defense and cellular signaling. Oxidative stress plays a central role in variety of pathological conditions ranging from inflammatory conditions to cancers.

Maintaining a reducing environment within the cell is essential for maintaining structural and functional integrity of macromolecules such as proteins, lipids and DNA. Oxidative stress chemically may be described as either a large increase in the "cellular reduction potential" (i.e.becoming less negative) or a large decrease in the "reducing capacity of cellular redox couples" (Schafer and Buettner, 2001). The effect of such a stress depends on its magnitude and may range from initiation of antioxidant defense to triggering apoptosis or even necrosis (Nkabyo et al., 2002, Schafer and Buettner, 2001, Attene-Ramos et al., 2005).

Endogenous pro-oxidant and antioxidant mechanisms:

Oxidative stress within cells is a result of an unfavorable tilt in the fine balance between pro-oxidant species and antioxidant mechanisms. The sources and importance of major players in this balance within normal cells is highlighted in the table below:

Pro-oxidants	
Oxidant	**Description and mechanism of generation**
O_2^- superoxide anion	This radical is generated by one electron reduction of molecular oxygen. Superoxide radicals may be formed during electron transfer in the mitochondria, uncoupling of eNOS or by enzymes such as NADPH oxidase, xanthine oxidase and cytochrome P-450. Destroys Fe-S centers by oxidation (Scandalios, 2005, Fortuno et al., 2005).
H_2O_2, hydrogen peroxide	This is a non-radical ROS. Hydrogen peroxide in cells is formed by dismutation of superoxide, β-oxidation and proton induced decomposition of superoxide by monooxygenases and oxidases (Scandalios, 2005, Fortuno et al., 2005).

$^{\bullet}$OH hydroxyl radical	The hydroxyl radical is formed during the reaction of H_2O_2 with O_2^- (Haber-Weiss) or Fe^{2+} (Fenton). Highly reactive with all macromolecules (Scandalios, 2005, Fortuno et al., 2005).
OONO$^-$, peroxynitrite	Strong pro-oxidant molecule formed by the inactivation of NO by O_2^- (Scandalios, 2005, Fortuno et al., 2005).
HOCl, hypochlorous acid	Hypochlorous acid is a strong oxidizing agent that can attack a variety of biomolecules and is generated by activated neutrophils. Myeloperoxidase catalyses the oxidation of halides by H_2O_2 to generate hypochlorous acid which exists in equilibrium with the hypochlorite ion (Halliwell et al., 1988).
RO•, alkoxy **ROO•, peroxy radicals**	These radicals are produced from lipid and protein intermediates in the presence of oxygen radicals by radical addition to double bonds or hydrogen abstraction. Lipid forms participate in lipid peroxidation reactions (Hawkins and Davies, 2001, Blair, 2001)

Enzymes with primary antioxidant role

Enzyme	**Subcellular location and reaction catalysed**
Superoxide dismutase	Cytosol (Cu/ZnSOD); mitochondrion (MnSOD) $2^{\bullet}O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$
Catalase	Peroxisome; cytosol; mitochondria $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$
Glutathione reductase	Mitochondrion; cytosol

	$2\ GSH + NADP^+ \leftrightarrow GSSG + NADPH + H^+$
Glutathione-S transferases	Cytosol; microsomal
	$RX + GSH \leftrightarrow HX + R\text{-}S\text{-}G$
	R may be an aliphatic, aromatic or heterocyclic group; X may be a sulfate, nitrile or halide group.
Peroxidases (non-specific)	Cytosol; cell membrane bound
	donor + H_2O_2 → oxidized donor + 2 H_2O
Dehydroascorbate reductase	Cytosol
	2 GSH + dehydroascorbate $\leftrightarrow$ GSSG + ascorbate
Reference: (Scandalios, 2005)	

Oxidative stress and NSAIDs - "the paradox"

Mechanistic studies on the enzyme cyclooxygenase suggest that the conversion of arachidonic acid to the prostaglandin precursor PGH_2 occurs through a two-step process. The first step involves the cyclooxygenase reaction where in arachidonic acid is converted into its cyclic endoperoxide PGG_2. PGG_2 is then converted to PGH_2 through a second peroxidase reaction that is also catalyzed by the same enzyme. This reaction involves the release of a superoxide radical. NSAIDs were believed to exert their anti-inflammatory effects by inhibiting both prostaglandin synthesis and superoxide radical generation (Palmer, 1995).

However, studies on NSAID toxicity have shown that NSAIDs cause a paradoxical increase in oxidative stress in animals, in both the kidney and gastric mucosa (Basivireddy et al., 2004, Basivireddy et al., 2003). The oxidative stress seen in these studies, involve both excessive generation of pro-oxidants and impairment in defense responses. The various mechanisms, which are thought to play a role in such process, are as follows:

Generation of free radicals:

1. Mitochondrial uncoupling:

The possibility of mitochondria as a source contributing to the development of oxidative stress initially came to light with electron microscopy studies show ballooning and vacuolization of mitochondria, features suggestive of uncoupling of oxidative phosphorylation within an hour of dosing with indomethacin (Somasundaram et al., 1997).

2. Neutrophil infiltration:

Neutrophil adhesion to the vascular endothelium has been described as one of the earliest events in NSAID mediated gastric injury. Reduction in mucosal injury in neutropinic rats and in animals treated with antibodies to β2 integrin CD-18 suggests the pivotal role of this event (Wallace et al., 1993). Intravital video microscopy studies to monitor leucocyte-endothelial cell adhesion show an increase in neutrophil endothelial adhesion on treatment with asprin or indomethacin. Leukotriene B4 receptor antagonists effectively decreased these interactions (Kurose et al., 1996). Interactions between leukocyte and endothelial cells are thought to lead to gastric microvessels occlusion and ischaemic/hypoxic endothelial-epithelial cell damage (Fiorucci et al., 1999). However, even though neutrophil infiltration is a salient feature of NSAID induced renal toxicity (Basivireddy et al., 2004), such interactions between neutrophils and endothelial cells have not been reported in the kidney.

3. Role of peroxidases:

The stomach, intestine and liver are tissues demonstrating high levels of peroxidase activity. Furthermore, both the intestine and kidney demonstrate significant neutrophil infiltration with high activity of myeloperoxidase in response to administration of NSAIDs (Basivireddy et al., 2004, Menozzi et al., 2006). Indomethacin and horseradish peroxidase/H_2O_2 in the

presence of Fe^{3+} causes peroxidation of lipids from liver microsomes in a time dependent manner (Miura et al., 2002). Protection offered by the enzyme superoxide-dismutase against both lipid peroxidation and Fe^{3+} reduction suggests the presence of the superoxide anion (Miura et al., 2002). Electron spin resonance studies also indicate the presence of an indomethacin free radical in this process (Miura et al., 2002, Chattopadhyay et al., 2006).

4. **Metabolism of NSAIDs to reactive radicals:**

The role of free radicals generated from indomethacin or its metabolites in causing an altered redox balance in tissues has been previously reported (Miura et al., 2002). Metabolism of O-desmethyl N-deschlorobenzoylindomethacin (DMBI) has been noted in activated neutrophils to give rise to reactive iminoquinone intermediates (Ju and Uetrecht, 1998). These reactive intermediates have been quoted to play a major role in idiosyncratic drug reactions such as agranulocytosis induced by indomethacin.

Impairment of endogenous antioxidant defense

The redox status of a cell or tissue is a reflection of the existing balance between pro-oxidants and antioxidants. An impaired antioxidant defense mecanisim is often contributory to oxidative stress seen. In the case of NSAIDs this may be exemplified by studies on indomethacin induced hepatotoxicity in rats, which showed depression in expression levels of antioxidant enzymes glutathione reductase, superoxide dismutase and glutathione transferase(LaFramboise et al., 2006). Administration of aspirin or nimusulide in rats reduces the activity of catalase, superoxide dismutase and glutathione-S-transferase in the intestine (Nair et al., 2006). A decrease in glutathione reductase and glutathione-s-transferase activity is also noted with ketoprofen (Nieto et al., 2002). The comman factors seen among these NSAIDs appears to be a net reduction in the levels of reduced glutathione which is a predisposing condition to the devlopment of oxidative

stress (Nieto et al., 2002, Nair et al., 2006). Previous studies done in our lab also show similar results and are in agreement with the results above.

Oxidative stress and renal dysfunction:

Oxidative stress plays an important role in the kidney, not only in mediating pathological changes, but also in regulating normal physiological adaptations. The impact of oxidative stress in the kidney may arise from an increased generation of ROS, a decrease in the antioxidant defense mechanisms or by a combination of both.

Initial interest in ROS as vasoactive mediators focused on superoxide free radical ($O_2^{-\bullet}$) and its interaction with nitric oxide (NO) as a mechanism to explain endothelial dysfunction and renal vasoconstriction (Laursen et al., 1997). Superoxide radicals generated by NADPH oxidase, under the influence of angiotensin II, modify tubulovascular crosstalk in the outer medulla. The effects of chronic angiotensin-II infusion include renal cortical hypoxia, hypertension, increased NADPH-oxidase activity, reduced expression of endothelial cell superoxide dismutase and inefficient usage of O_2 for tubular transport (Welch et al., 2005). It is interesting to note that these effects of angiotensin II are blunted or prevented by Tempol, a well-validated spin trap for superoxide (Welch et al., 2005, Krishna et al., 1996, Konorev et al., 1995).

It is well known that reactive oxygen species (ROS) are proximal effectors in multiple signaling pathways implicated in organ dysfunction or damage (Wu, 2006, Fialkow et al., 2007). Downstream effects from ROS involve the activation of pro-inflammatory pathways such as nuclear factor kappa B (NFκB), activator protein-1 (AP-1) and peroxisome proliferator-activated receptor (PPARs) (Fialkow et al., 2007, Wilcox and Gutterman, 2005). The activation of these signaling cascades result in generation of secondary mediators involved in cellular adhesion

(mediated by Intracellular adhesion molecule-1 and vascular cell adhesion molecule-1), extracellular matrix remodelling (matrix metalloproteinases), inflammation (cyclooxygenase and phospholipase A_2) and cytokine production (epidermal growth factor). These substances in association with their more proximal inducers perpetuate organ damage (Wilcox and Gutterman, 2005).

The central role of ROS in diffrent renal pathologies stand as a testimony to the mechanism described above. Increases in glomerular levels of H_2O_2 and $OH^{\bullet}$ have been observed after treatment with puromycin aminonucleoside in rats, a standard model for human minimal-change glomerulopathy (Gwinner et al., 1997, Kawaguchi et al., 1992)**.** Similar evidence of the role of oxidative stress has been established in mesangioproliferative GN give expansions, membranous GN and segmental necrotizing GN and animal models of these conditions(Gwinner and Grone, 2000) . Reactive oxygen species have been implicated in several other renal diseases, including diabetic nephropathy, hypertensive nephropathy, ischemia reperfusion injury and transplant rejection (Singh et al., 2006, Nath and Norby, 2000, Johnson and Weinberg, 1993, Tan et al., 2007, Ha and Lee, 2005).

ANTIOXIDANTS

Generation of reactive oxygen species is a ubiquitous phenomenon in all-living organisms. However reactive oxygen species can induce peroxidation of cellular lipids, proteins and DNA, which is deleterious to the organism. In a bid to reduce the impact of such reactive molecules cells have evolved various types of antioxidant and repair mechanisms.

Antioxidants may be broadly defined as agents that limit the deleterious effects of free radical-induced oxidant reactions. Some of the antioxidants that have an important role in health and disease are as follows:

Antioxidants utilized by the body	
Endogenous antioxidants	**Exogenous/dietary antioxidants**
Enzymes and their substrates: Superoxide dismutases (SOD): • MnSOD mitochondrial • CuZnSOD cytosolic Catalase Glutathione peroxidase Metal ion sequestration: Metallothioneins Transferrin Lactoferrin	Naturally occurring dietary antioxidants Vitamin E Vitamin C β-Carotene Flavinoids and plant penolics Food additives serving as antioxidants Propyl gallate Butylated hydroxyanisole Butylated hydroxytoluene

Antioxidants can be classified based on mechanism of action as:

1. Preventive antioxidants: These antioxidants reduce the rate of initiation of peroxidation. Examples of preventive antioxidants are:
    i. endogenous: catalase, peroxidases and metallothionins
    ii. exogenous: diethylenetriaminepentaacetate (DPTA) and ethylenediamine-tetraacetate (EDTA)
2. Chain breaking antioxidants: These antioxidants stop the propagation of peroxidation chain reactions. Examples of chain breaking antioxidants are:
    i. endogenous: superoxide dismutase and urate (aqueous phase) and vitamin E (lipid phase).

ii. exogenous: polyphenols and aromatic amines

ZINC

Zinc is a metal belonging to the class IIb family on the periodic table, with an atomic number of 30 and an average atomic weight of 65.38amu.

The tissue concentration of zinc is 10 to 30 μg/g rat tissues (Onosaka and Cherian, 1982). The levels are very high in the prostate. The zinc in the cells mainly distributes in the cytoplasam and is protein-bound (Cousins, 1986). Zinc supplementation results in accumulation in four organs the pancreas, liver, intestine and the kidneys in descending order (Onosaka and Cherian, 1982).

The levels of zinc in plasma are maintained at approximately 1mg/ml (Wise, 1995). The levels rise after administration of I.V. zinc and return to normal in 12 hours. Actinomycin D maintains the increase in plasma zinc for 24 hours (Richards and Cousins, 1975).

Zinc as an antioxidant

Zinc is redox inert in biological systems. The antioxidant effects of zinc do not fall clearly into the previously mentioned classifications. One of the factors that make it a unique in its antioxidant role is that there is no direct interaction ever shown between zinc and oxidant species (Powell, 2000). There are several proposed mechanisms, which attempt to explain the indirect antioxidant effects of zinc, some of which are as follows:

Possible mechanisms for the antioxidant effects of zinc	
Proposed mechanism	Reference
• Protection against vitamin E depletion	(Bunk et al., 1989, Kim et al., 1998)
• Stabilization of membrane structure	(Bray and Bettger, 1990)
• Restriction of endogenous radical production	(Bray and Bettger, 1990)
• Essential structural component of the enzyme CuZnSOD	(Marklund, 1982)
• Maintenance of tissue concentrations of metallothionein	(Cousins, 1985)
• Stabilization of protein thiols	(Gibbs et al., 1985)

The antioxidant effects of zinc may be divided into acute and chronic effects (Powell, 2000). The acute effects of zinc supplementation are a consequence of the protection conferred to sulfhydrals and a reduction of redox active transition metals, such as iron and copper. The chronic effects of zinc supplementation include the induction of metallothioneins, a group of low molecular weight metal binding proteins.

The antioxidant role of zinc attributed to its ability to bind and stabilize protein thiols was initially investigated in δ-aminolevulinate dehydratase, an enzyme that depends on its thiol groups for activity. The thiol groups were stabilized by Zn^{2+} and were found to be less prone to oxidation (Gibbs et al., 1985).

Transition metals like iron and copper associate with high or low molecular weight cellular components such as nucleotides, peptides, proteins or DNA (Chevion, 1988, Czapski et al., 1984). These associations form loci of bound metals undergoing repeated redox cycling of metals thereby inducing site-specific free radical generation (Chevion, 1988). By virtue of similarities in

coordination chemistry, it is proposed that zinc can effectively compete with transition metals for critical binding sites (Cotton and Wilkinson, 1972). Experimental evidence supporting such antagonism is most clearly seen by the competition between zinc and copper for binding to heme proteins (Hegetschweiler et al., 1987). The displaced transition metals either bind to less critical sites or undergo hydrolytic polymerization in the cytosolic compartment (Spiro et al., 1967). Zinc has been noted to antagonize iron-mediated, xanthine/xanthine oxidase- induced peroxidation of RBC membranes (Girotti et al., 1986). Zinc has been established as a cardio-protective agent after myocardial ischemia. Perfusion of ischemic myocardium with a zinc-containing buffer induces the removal and excretion of copper from the myocardial tissue (Powell et al., 1999). This is associated with improvements in post-ischemic myocardial function and a reduction in the synthesis of ROS.

Metallothioneins are low molecular weight proteins that are induced by the activation of metal responsive elements in by high environmental concentrations of zinc or other transition metals (Powell, 2000). Conditions of copper or cadmium overload can readily induce the displacement of zinc from these proteins and hence confer protection against their toxicity (Shaw et al., 1991). It is also proposed that metallothioneins are anti-apoptotic, an effect mediated by NFκB (Abdel-Mageed and Agrawal, 1998). Rate constants measured for reactions between metallothioneins and reactive oxygen species (hydroxyl radicals and superoxide radicals) suggest a sacrificial oxidation of up to 20 cysteine sulphydral groups per molecule (Thornalley and Vasak, 1985). The sulphydral groups that are lost to ROS can be later regenerated by reducing agents like glutathione (Thornalley and Vasak, 1985, Abel and de Ruiter, 1989).

Studies on zinc deficiency in both humans and animals have given us further insights into the importance of zinc as an antioxidant. Chronic zinc deficiency in rats is known to be associated with an increase in conjugated dienes and malondialdehyde production in the liver (Sullivan et

al., 1980). Experiments by Bray and collogues concluded that zinc deficiency evoked NADPH oxidase and cytochrome P450-induced increases in lipid peroxidation in both the lung and the liver (Bray et al., 1986). Other studies have demonstrated similar results in other tissues (Burke and Fenton, 1985, Oteiza et al., 1995).

Finally zinc has an important role in the structure and stabilization of important antioxidant enzymes. Copper, zinc-superoxide dismutase catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide. The latter and other hydroperoxides are subsequently reduced by the selenoenzyme glutathione peroxidase (GPx) (Klotz et al., 2003).

Intracellular signaling and oxidative stress: the role of zinc

Zn^{2+} is capable of inducing a stress response in terms of the stimulation of expansions when used for 1st time MTF-1 dependent transcription and activation of stress responsive signal cascades such as expansions when used for 1st time MAPK and PI3K/AKT.

Zn deficiency-triggered oxidative stress could affect cell signaling, including (Powell, 2000):

1. Transcription factors containing Zn finger motifs
2. Other oxidant-sensitive transcription factors (NF-κB and AP-1).

The Zn finger motif in the Zn finger transcription factors is mainly a DNA-binding domain. Cysteine residues coordinate the Zn ion folding structural domains that participate in intermolecular interactions. Oxidative stress can impair the DNA-binding activity of Zn finger transcription factor, by oxidizing the cysteine residues and therefore altering the secondary structure of the protein (Oteiza and Mackenzie, 2005).

Furthermore zinc deficiency is associated with higher pro-oxidant levels that alter several signal transduction pathways. AP-1 and NFκB are activated by an increase in cellular oxidants in a background of zinc deficiency (Oteiza and Mackenzie, 2005).

The role of zinc in regulating redox state

Zinc/cysteine coordination environments in proteins are redox active. Oxidation of the sulfur ligands mobilizes zinc, while reduction of the oxidized ligands enhances zinc binding, providing redox control over the availability of zinc ions. Some zinc proteins are redox sensors, in which zinc release is coupled to conformational changes that control varied functions such as enzymatic activity, binding interactions and molecular chaperone activity. Whereas the released zinc ion in redox sensors has no known function, the redox signal is transduced to specific and sensitive zinc signals in redox transducers. Released zinc can bind to sites on other proteins and modulate signal transduction, generation of metabolic energy, mitochondrial function, and gene expression (Powell, 2000).

Zinc and NSAID-mediated renal damage:

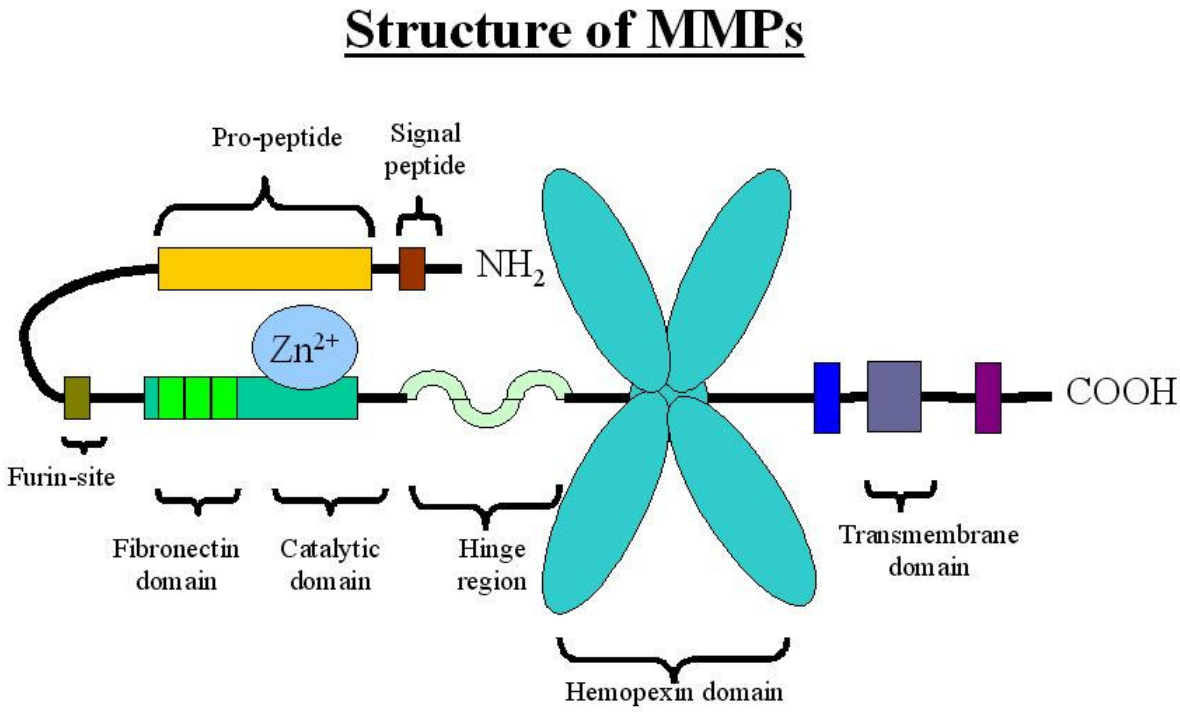
Work done in the past at our laboratory has shown that indomethacin, when administered at a dose of 20mg/kg to male rats, is known to produce oxidative stress, alter membrane lipid composition and induce changes in the microscopic architecture of the kidney. Oxidative stress induced in the above manner was mitigated by L-arginine a nitric oxide donor (Basivireddy et al., 2004). Mitochondrial dysfunction and altered balance of pro- and antioxidant enzymes have been proposed as possible mechanisms for these changes (Basivireddy et al., 2004, Varghese, 2006). Preliminary work done in our laboratory has shown that zinc, protected against such effects (unpublished data). It resulted in a reversal of pro-oxidant changes and increased the activities of antioxidant enzymes.

MATRIX METALLOPROTEINASES (MMPS)

Matrix metalloproteinases are a group of proteins collectively referred to as matrixins that participate in the degradation of the extracellular matrix (ECM) (Visse and Nagase, 2003). In addition to the ECM, these proteases also degrade several other molecules that include hormones, growth factors, cytokines and other MMPs and hence contribute to the maintenance of the extracellular environment (Visse and Nagase, 2003, Folgueras et al., 2004).

Structure of MMPs

Shown below is the general structure of MMPs.



Classification of MMPs

This family consists of 44 different MMPs among which 23 are present in humans (Visse and Nagase, 2003, Folgueras et al., 2004). Sequence homology to collagenase 1 (MMP-1), the

cysteine switch motif, PRCGXPD that is essential to maintain the protein as an inactive zymogen, and the zinc-binding motif, HEXGHXXGXXH at the catalytic domain, are signatures used to assign proteins to this family. The only exception to this rule is MMP-23, which lacks the cysteine switch motif, but however maintains structural similarities to MMP-1 (Visse and Nagase, 2003).

Vertebrate MMPs can be divided into six groups based on substrate specificity, sequence similarity and domain organization as follows:

I. Classification of MMPs based on substrate specificity and structural features

Class	MMPs	Substrates and Structural Features
Collagenases	1, 8, 13 and 18	Cleave collagens types I, II and III
Gelatinases	2 and 9	Denatured collagen, laminin and gelatin Fibronectin repeats in catalytic domain
Stromelysins	3, 10, 11	proMMP-1
Matrilysins	7, 26	Lacks a hemopexin domain Pro-α-defensin, Fas-ligand, E-cadherin and pro-TNF-α
MT-MMPs	14[1], 15[1],16[1], 24[1], 17[2] and 25[2]	ProMMP-2*, collagen type I, II, III [1] type-1 transmembrane proteins [2] GPI anchored proteins
Other MMPs	12, 20,22, 23, 28	Elastin, amelogenin

*All members of MT-MMP class except MT-MMP-4; MT-MMP: Membrane type MMP

Gelatinases

The key focus of this study rests on the gelatinases, MMP-2 and MMP-9. They are zinc-dependent endopeptidases, which are involved, in a wide variety of physiological and pathological processes (Lelongt et al., 2001, Visse and Nagase, 2003).

MMP-2 and MMP-9 are similar in terms of structure and substrate specificity. The nascent gelatinases have a N-terminal signal sequence called as the pre -domain that directs the protein to the endoplasmic reticulum. The pre-domain is followed by a pro-domain that maintains enzyme latency. Cleavage of the pro-domain exposes the catalytic domain that contains cysteine repeats, collagen II binding fibronectin type II inserts and a conserved zinc-binding region. The catalytic regions are connected by a hinge region to four hemopexin-like domains that, among other things, mark the enzyme for specific tissue inhibitor of metalloproteinase (TIMP) recognition. The gelatinases share a wide array of substrates, which include basement membrane components such as type IV and type V collagens, aggrecan, elastin and gelatins (denatured collagens), as well as non-basement membrane proteins, such as IL-1β, substance P, mylein basic protein, amyloid β peptide and pro-transforming growth factor β (Lelongt et al., 2001). Cleavage of non-basement membrane proteins is more so with MMP-9 than MMP-2. Both MMP-2 and MMP-9 require zinc and calcium for their activity and bind at a ratio of 2 zinc and 3 calcium ions per subunit (Lelongt et al., 2001, Visse and Nagase, 2003).

MMP-2 and MMP-9 show differences in molecular weight, expression patterns, regulation, inhibition by TIMP and pathways of activation. This is the basis for differences in the gelatinase profiles observed in different pathologies (Lelongt et al., 2001). The differences between the two gelatinases MMP-2 and MMP-9 are highlighted in the table below.

Gelatinases and their Characteristics		
Characteristics	**MMP-2**	**MMP-9**
Synonyms	• EC 3.4.24.24 • 72 kDa gelatinase • Gelatinase A	• EC 3.4.24.35 • 92 kDa gelatinase • Gelatinase B
Gene location (Humans)	16q13-q21	20q11.2-q13.1
Molecular Weight (pro-forms)	72kD	92kD
Molecular Weight (active forms)	64kD	82, 67 and 64kD
Co-factors	4 Ca and 2 Zn ions per subunit	3 Ca and 2 Zn ions per subunit
Activators	MT-MMP, trombin, urokinase	Cathepsin, trypsin, α-chymotrypsin, stromelysin, colalgenase-I, matrilysin, mast cell chymase, MMP-2, trypsin
Regulation	Mainly post-transcriptional	Mainly transcriptional
Expression in the kidney	Constant low level expression	Highly inducible

Regulation of gelatinases

Transcriptional regulation:

One of the remarkable features of matrixins is the inducible nature of many of their genes. Studies on the promoter region of MMP-9 reveal motifs that are homologous to the binding sites for activator protein-1 (AP-1), PEA3/ets, NF-kB and transcription factor SP-1(Sato and Seiki, 1993). Induction of MMP-9 gene transcription has been observed with 12-O-tetradecanoyl-phorbol-acetate and tumor necrosis factor (TNF) alpha (Sato and Seiki, 1993). Ultravoilet B irradiation up-regulates MMP-9 expression in human dermal fibroblasts (Kut et al., 1997). This was reported to be a consequence of activation of stress-activated protein kinase (JNK-2) through

the generation of reactive oxygen species and lipid peroxidation by the ion-driven Fenton reaction (Wang and Kochevar, 2005).

Other mechanisms of gelatinase induction include increased MMP-9 mRNA stability induced by transforming growth factor-beta1 (TGF-1) (Sehgal and Thompson, 1999), stabilization of the pro enzyme form of MMP-2 by TGF-1 (Sehgal and Thompson, 1999) and regulation of translational efficiency (Jiang and Muschel, 2002).

Proenzyme activation:

MMPs can be activated by proteinases in vivo or by chemical agents such as thiol-modifiying agents (4-aminophenylmercuric acetate, $HgCl_2$ and N- ethylmaleimide), oxidized glutathione, SDS, chaotropic agents and reactive oxygen species in vitro (Visse and Nagase, 2003). These agents are thought to act by disturbing the cysteine zinc interaction of the cysteine switch following which the propeptide segments are removed in a stepwise manner (Van Wart and Birkedal-Hansen, 1990).

ProMMP-2 is resistant to activation by general proteinases. The major pathway of activation of proMMP-2 takes place on the cell surface and is mediated by membrane type matrix metalloproteinases (MT-MMPs) (Visse and Nagase, 2003). All members of the MT-MMP family except MT-MMP4 have been demonstrated to activate proMMP-2. Studies show that the activation of proMMP-2 requires both active MT-MMP-1 and TIMP-2 bound MT-MMP-1. The TIMP-2 bound MT-MMP-1 acts as a receptor for proMMP-2 (Butler et al., 1998). Pro-MMP-2 binds to the free C-terminal domain of TIMP-2 through its hemopexin domain that is assumed to localize it near an active MT-MMP-1 (Wang et al., 2000).

Plasmin is an important activator of proMMP-9 in vivo (Pepper, 2001, Kaur et al., 2004, Lijnen, 2001). Plasmin is generated from plasminogen by tissue plasminogen activator bound to fibrin and urokinase type plasminogen activator bound to a specific cell receptor (Pepper, 2001). The membrane localization of both plasminogen and urokinase plasminogen activator assists localized pro-MMP activation and ECM turnover (Pepper, 2001, Collen, 2001). MMP-2 is also activated by the cell surface associated uPA /plasmin system, whereas soluble plasmin degrades it (Mazzieri et al., 1997).

MMP-3 can activate proMMP-9, and MMP-3 and MMP-10 can super activate procollagenase, thereby generating collagenase with higher specific activity (Knauper et al., 1993, Nagase, 1997).

Inhibitors:

Inhibitors of MMP activity may be classified as endogenous inhibitors like tissue inhibitors of metalloproteinases (TIMPs), which are specific inhibitors of MMPs, and as exogenous inhibitors like drugs, which act by chelating Ca^{2+} and Zn^{2+}. The tetracycline group of antibiotics, for example, inhibits MMP activity by virtue of its chelating ability, a property that is entirely distinct from their antibacterial actions (Woessner, 1999).

Some of the other important, but non-specific, inhibitors of MMP activity include tissue factor pathway inhibitor-2 (TFPI-2), thrombospondin 1 and 2, alpha-2 macroglobulin and reversal-inducing cysteine rich protein with Kazal motifs (RECK) (Visse and Nagase, 2003). TFPI-2 is a serine protease inhibitor that shares structural a homology with TIMP and inhibits MMP-1, -2, -9 and –13. TFPI appears to bind and co-precipitates with MMPs suggesting sequestration as a possible mechanism (Herman et al., 2001).

Thrombospondin-2 can regulate MMP-2 by forming a complex that facilitates scavenger-receptor mediated endocytosis. Similarly, thrombospondin-1 has been shown to inhibit the activation of MMP-2 and MMP-9 pro-forms and also to modulate MMP-2 production (Egeblad and Werb, 2002). Alpha-2-macroglobulin is a 772kD plasma proteinase inhibitor synthesized mainly in the liver by hepatocytes. Even though TIMPs are present, α2-macroglobulin is thought to be the major MMP inhibitor in plasma. The mechanism of inactivation of MMPs by α2-macroglobulin involves the presentation of a cleavable bait region to the MMP which, when proteolytically cleaved, traps the proteinase within the α2-macroglobulin that later becomes covalently attached by transacylation (Baker et al., 2002). RECK is an 110kD glycoprotein that contains serine -proteinase-inhibitor -like domains and associates with the cell membrane through a GPI anchor. It is widely expressed in normal human tissues but its levels appear to be low in several tumor cell lines. Over-expression of RECK has been shown to post-transcriptionally downregulate MMP-9 and inhibit the cellular activation of MMP-2. $RECK^{-/-}$ mice that die at E10.5 and show severe disruption of mesenchymal tissue and organogenisis underline the importance of this protein (Oh et al., 2001). However, embryos of $RECK^{-/-}$, $MMP\text{-}2^{-/-}$ double knock-out mice show improved vascular development, larger body size and survive till E11.5 (Oh et al., 2001).

Tissue inhibitor of matrix metalloproteinases (TIMPs):

The tissue inhibitors of metalloproteinases, thus far, consist of four family members, TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMPs inhibit the activity of MMPs by forming tight non-covalent complexes in a 1:1 stoichiometric ratio with MMPs (Visse and Nagase, 2003).

TIMPs are smaller proteins in comparison to the MMPs and have molecular weights ranging from 21 to 29 kDa and are variably glycosylated (Brew et al., 2000). Structurally, TIMPs are cysteine-rich proteins, which contain three disulphide bonds. They are generally compromised of two domains, the larger N-terminal domain associated with MMP inhibition and a smaller C-terminal domain (Brew et al., 2000, Visse and Nagase, 2003). The characteristics of individual members of the TIMP family are given in the table below.

Characteristics of members of the TIMP family

Characteristics	**TIMP-1**	**TIMP-2**	**TIMP-3**	**TIMP-4**
Synonyms	Erythroid-potentiating activity Fibroblast collagenase inhibitor	CSC-21K	Protein MIG-5	Cardiac inhibitor of metalloproteinase
Length of peptide	207 AA	220 AA	211 AA	224 AA
Molecular weight	28kD	21kD	24/27kD	22kD
Gelatinase inhibition	Pro-MMP-9	Pro-MMP-2	Pro-MMP-2&9	Pro-MMP-2
Localization	Soluble	Soluble/Cell surface	ECM	Soluble/Cell surface
N-glycosylation sites	2	0	1	0

TIMP-1 and TIMP-2 are the best-characterized members in this family. These have erythroid-potentiating activity, growth stimulating effects and regulate development and apoptosis in

various cells in addition to its MMP inhibiting effects (Stetler-Stevenson et al., 1992, Hayakawa et al., 1992, Barasch et al., 1999).

Gelatinases and the kidney

The expression profiles of MMPs and TIMPs in the kidney are complex and species dependent. MMP-2, -3, -9, -13, -14, -24, -25, -27, -28 and TIMP-1, -2, and -3 are all expressed in the kidney (Catania et al., 2007).

The profile of gelatinase expression in the kidney has been well characterized in both rats and humans. MMP-2 is the major gelatinase expressed in the glomerulus, proximal tubules and distal tubules in most species, including rats (Tomita et al., 2004, Inkinen et al., 2005, Schaefer et al., 1996). The expression of MMP-9 is confined to the glomerulus and initial parts of the PCT (Inkinen et al., 2005, Schaefer et al., 1996, McMillan et al., 1996).

TIMP-1 is expressed in the glomerulus in both humans and rats (Tomita et al., 2004). There is no clear evidence as yet about the normal pattern of expression of TIMP-2 and TIMP-3 in normal rat kidneys (Catania et al., 2007).

The role of MMPs in acute kidney injury in pathologies related to oxidative stress, such as ischemia-reperfusion, has been experimentally established previously (Ziswiler et al., 2001). Studies with a single dose of 30mg/kg BB-94, a broad spectrum MMP inhibitor, reduced proteinuria (Ermolli et al., 2003) but not the increase in serum creatinine associated with ischemia-reperfusion injury (Ziswiler et al., 2001). MMP-2 and -9 were increased in renal tubules and in the interstitium after 1 to -3 days of reperfusion, following 52 min of ischemia in rats (Basile et al., 2004). Other studies with 30 minutes of ischemia followed by 60 minutes of

reperfusion were also associated with increased expression of MMP-2 and -9 and TIMP-2 in glomeruli, and a decrease in TIMP-1 (Caron et al., 2005).

Gelatinases and NSAIDs

Anti-inflammatory agents, such as indomethacin, have been used to reduce invasion of cancer cell lines (Ackerstaff et al., 2007, Diament et al., 2006, Wang et al., 2005) . Cells over-expressing COX-2 acquire increased invasiveness and angiogenic ability by activation of vascular endothelial growth factor (VEGF), urokinase like plasminogen activator (uPA) and matrix metalloproteinase-2 (MMP-2). NS-398 and indomethacin are known to inhibit these effects (Li et al., 2002). NSAIDs are also known to suppress ERK/Sp-1 mediated transcription which in turn results to decreased MMP-2 transcription (Pan and Hung, 2002).

Indomethacin is also known to prolong gestation in rabbits by decreasing MMP-2 and –9 activities and increasing TIMP-1 levels in the cervix (Fortson et al., 2006). Studies also demonstrate induction of MMP-9 by urokinase-type plasminogen activator (uPA) in THP-1 monocytes is via a pathway involving MEK1-ERK1/2-mediated activation of cytosolic PLA_2 and eicosanoid generation. This suggests an important role for ecosinoids in monocyte activation and migration induced by uPA and MMP-9, an effect that can be inhibited by NSAIDs (Menshikov et al., 2006).

Indomethacin toxicity is associated with gastric ulceration. This process is mediated by reactive oxygen species and is associated with a reduction in MMP-2 transcription and translation both of which are reversed by antioxidants such as melatonin (Ganguly et al., 2006) . Data on the role of MMPs in NSAID-mediated renal toxicity is scarce.

Gelatinases and oxidative stress

Treatment of mesangial cells with pro-inflammatory cytokines resulted in an increase in the biosynthesis of NO and superoxide, the precursors of the highly potent pro-oxidant $ONOO^-$, and an associated increase in MMP-2 activity (Okamoto et al., 1997). It has also been demonstrated that superoxide, hydrogen peroxide or $ONOO^-$ could enhance gelatinolytic activity of unpurified MMP-2 derived from smooth muscle cells and their media (Rajagopalan et al., 1996). Similar experiments with hydrogen peroxide or superoxide generating systems in cultured rat fibroblasts showed enhanced activity of MMP-2, -9, and –13 (Siwik et al., 2001). Superoxide is believed to play a greater role than NO in this process as elimination of the former failed to activate purified form of MMP-8 (Rajagopalan et al., 1996).

The mechanism of activation of MMPs by reactive oxygen and nitrogen species is thought to involve the oxidation of sulfhydryl groups located on cysteine residues that form the autoinhibitory peptide domain, thus influencing its interaction with the coordinated Zn^{2+} at the catalytic site (Owens et al., 1997). This could result in changes that may induce, modulate or inhibit enzyme activity in response to the redox state within the cell (Owens et al., 1997).

The exact nature of post-translational modifications of MMPs resulting in their activation or inactivation still remains a matter of debate. Experiments have shown that minimal concentrations of $ONOO^-$ (1-20μM) activate MMP-1, -8 and –9 without removal of the autoinhibitory pro-peptide domain (Schulz, 2007). In the presence of normal cellular levels of glutathione, $ONOO^-$ causes S-glutathiolation of the cysteine-containing PRCGVPD sequence within this domain resulting in activation of the enzyme and a modification of its size, that is difficult to detect on standard SDS PAGE but may be detected by autoradiography or mass spectrometry (Okamoto et al., 2001). In contrast, higher concentration of $ONOO^-$ is known to

inactivate MMP-2 activity, possibly by nitration of tyrosine residues in the sequence. Similar studies on MMP-9 show an increase in enzyme activity coinciding with evidence of S-nitrosylation of the pro-peptide domain on mass spectrometry (Gu et al., 2002). Mass spectrometric analysis of MMP-2 on exposure to 3mM $ONOO^-$ have shown other extensive modifications, such as glutathiolation of Cys-65 and Cys-102, hydroxylation of Phe-583 and nitration of Tyr-244. In addition to activation of the enzyme, exposure to $ONOO^-$ may also alter the structural binding characteristics of cysteine-rich TIMPs, thus favoring an increase in MMP activity (Frears et al., 1996).

Zinc and MMPs

Matrix metalloproteinases are zinc-dependent endoproteinases. The zinc ion is present at the catalytic site and is essential for activity. Chelation of zinc causes an inhibition of MMP activity, which returns to normal in the presence of excess zinc (Boissier et al., 2000, Springman et al., 1995). These tests were, however, done in cancer cell lines, which were able to achieve high intracellular zinc concentrations as compared to normal cells.

Studies with cell lines indicate that zinc is able to suppress invasiveness of cell lines like PC-3 and SKRC-1 effectively. However, there was no effect on LNCaP cells. Zinc was found to effectively inhibit aminopeptidase and urokinase-type plasminogen activator (Ishii et al., 2001). These substances are known activators of MMPs. Zinc causes no changes in the activity of purified MMPs (Ishii et al., 2001).

- *The interactions between MMPs and zinc have been only explored with reference to its structural importance. Studies evaluating the indirect effects of zinc on MMPs are still limited and lie unexplored.*

-

- ***Hypothesis***

Indomethacin has been shown to alter microscopic architecture and induce oxidative stress in the kidney. Zinc has been shown to play a protective role against the above-mentioned changes. In view of these effects, we hypothesize that indomethacin-induced oxidative stress alters matrix metalloproteinase-antiproteinase balance in the rat kidney. We further hypothesize that zinc, by virtue of its antioxidant effects, may confer a protective effect in the maintenance of this balance.

- ***Aims and Objectives***

This study has been designed to achieve the following aims and objectives:

1. To study the effect of indomethacin on the activity of metalloproteinases (MMP) in the kidney of rats
2. To study the effect of indomethacin on the activity of tissue inhibitors of metalloproteinases (TIMP) in the kidney of rats
3. To assess the role of indomethacin -induced oxidative stress in the kidney in altering the renal protease-antiprotease balance.
4. To evaluate the effect of zinc in protecting against such effects.
5. To evaluate the collective effects of these alterations on renal function.

MATERIALS

The following chemicals have been used to carry out this thesis work:

1-[p-Chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid (indomethacin), acrylamide, ammonium persulfate, anti β-actin antibody, β-mercaptoethanol, bis-acrylamide, Tetrabromophenolsulfonephthalein (bromophenol blue), color burst molecular weight markers, N, N, N`, N`-tetramethyl-ethylenediamine (TEMED), sodium dodecyl sulphate were obtained from Sigma –Aldrich Chemicals, St. Louis U.S.A.

Calcium chloride, hydroxymethyl aminomethane hydrochloride (TRIS HCl), glycine, polyethylene glycol-4-tert-octylphenol ether (Triton X 100), sodium chloride, obtained from Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai.

Methanol was obtained from Qualigens fine chemicals.

1-butanol, gelatin, glacial acetic acid was obtained from E. Merck (India) Ltd.

Zinc sulphate was obtained from Sarabai Merck Ltd.

Polyoxyethylene monolauryl ether (Brij 35) was obtained from ICI America Inc.

Skimmed milk powder was obtained from Amul Corp. India

Anti-MMP-2, anti-MMP-9, anti-TIMP-1 and anti-TIMP-2 antibodies were obtained from Lab Vision Corp. U.S.A

Halothane IP (Fluothane) was obtained from Nicholas Parimal India Ltd.

- ***METHODOLOGY***

Animals

Male albino Wistar rats from the licenced animal house of the institution, weighing between 180-240 grams, were used for this study. The animals were fed with a commercially available standard rat chow diet ("Amrut" brand supplied by Sai Durga Feeds and Food). The animals were exposed to 12 hour light-dark cycle. The animals had free access to drinking water and food. The Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India, approved the experimental protocol (CPCSEA) (IAEC No 2/2006).

Drug dosage and preparation

Indomethacin was used at a dose of 20mg/kg body weight. The dose of indomethacin was calculated based on the weight of the animal. It was then dissolved in 1ml of 5% (w/v) sodium bicarbonate in distilled water by gentle warming.

Zinc sulphate was used at a dose of 50mg/kg body weight. The required dose of the drug was calculated based on the weight of the animal. It was then dissolved in 1ml of distilled water.

Protocol for drug administration

Protocol for indomethacin dose response studies: Experiments were done with batches containing five animals each, which were matched for age and weight. The control group was adminstered the veichle (5% bicarbonate) only. The other four animals in the group

were treared with increasing doses of indomethacin ranging from 10mg/kg to 60mg/kg respectively. All animals were sacrificed 24 hours later.

Protocol for indomethacin dose-response studies					
II. Time	***III. Treatment***				
0hr	Control 5% HCO_3^-	Indo 10mg/kg	Indo 20mg/kg	Indo 40mg/kg	Indo 60mg/kg
24hr	Sacrifice	Sacrifice	Sacrifice	Sacrifice	Sacrifice

Protocol to assess the effects of zinc: All experiments were done with batches of four rats each. The animals in each batch had similar weights and were separated into four groups designated as Control (group 1), Indo (group 2), Indo+Zn (group 3) and Zn alone (group 4) respectively.

Protocol to assess the effects of zinc			
Treatment group	Time (hr)		
	-2hr	0hr	24hr
Control	Water	5% bicarbonate	Sacrifice
Indo	Water	Indomethacin (20mg/kg)	Sacrifice
Indo+Zn	Zinc (50mg/kg)	Indomethacin (20mg/kg)	Sacrifice
Zn	Zinc (50mg/kg)	5% bicarbonate	Sacrifice

In all the experiments, the animals were fasted overnight. Animals in-group 3 and 4 were pretreated with zinc sulfate 50mg/kg body weight by gavage, where as the remaining two groups received an equal volume of the vehicle (water). 2 hours subsequently groups 2 and 3 were administered indomethacin at a dose of 20mg/kg body weight by oral gavage where as group's 1and 4 received only the vehicle (5% bicarbonate).

Processing of tissue

The animals were sacrificed by cervical dislocation, 24 hours later, under halothane anesthesia. Blood was drawn from the heart for estimation of serum urea and creatinine. Both kidneys were removed, decapsulated, washed with ice cold saline. cut into equal hemi-sections and snap-frozen in liquid nitrogen. The snap-frozen samples were kept at –70°C until further processing.

Experiments done using kidney homogenate

Preparation of renal homogenate

Snap-frozen renal tissue was washed twice with ice-cold saline and was minced into small pieces, using a pair of sharp scissors. The minced tissue was immediately homogenized in 2ml of homogenization buffer, containing 50mM Tris-HCl, sodium chloride and protease inhibitor PMSF (pH 7.4) (Heo et al., 1999) using a Potter-Elvehjem homogenizer at 7000 rpm for 1 minute (8-10 strokes). Renal homogenate obtained from the above process was centrifuged at 13,500g at 4^{o}C for 30 minutes to remove suspended cellular debris. The supernatant was used for zymography, reverse-zymography and western blots.

Gelatin zymography:

Supernatants from renal homogenates were loaded, at a standard concentration of 200µg protein per well, into 10% polyacrylamide gel containing 0.1% gelatin (Basile et al., 2004) and electrophoresed at 4°C. The gel was removed from the cassette and washed three times (30minutes each) with the wash buffer (2.5% triton X 100) at 4°C. The gel was then washed twice with water and incubated in a development buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Briji-35) for 16-18 hours at 37°C. Each gel was stained with 0.5% Coomassie blue G-250 in 30% methanol and 10% acetic acid for 40 minutes and destained for 15 minutes in 30% methonal/10% acetic acid. MMP activity was detected as areas of clearing in the gel, where the gelatin had been cleaved by the MMPs. The gels were digitized using a CCD frame digitizer system (Alpha-Innotech) and densitometric analysis of the cleared zones was done using AlphaEaseFC (Alpha-Innotech). Positive controls for MMP-2 and 9 were obtained from conditioned culture medium used for growing HT 1080 cell (human fibrosarcoma) (Inkinen et al., 2005). Medium was collected after 2-3 days of growth when the cells attained confluence and used as sources of MMP-2 and -9.

Protein quantification by Western blotting:

Supernatants from renal homogenates, containing 40µg protein, were denatured in the protein dissociation buffer containing 62.5mM Tris-HCl, 2% SDS, 10% glycerol, 0.004%

bromophenol blue and 5% β-mercaptoethanol for 5 minutes at 95°C, separated on 12% polyacrylamide gel at 200V and electroblotted to nitrocellulose membranes at 250 mA (Maric et al., 2004). The membranes were incubated in primary antisera and subsequently developed as per the manufacturer's protocol. The protein bands on the membranes were then digitized using a CCD frame digitizer (Alpha Innotech) and the bands were quantified by densitometry using the gel documentation system AlphaEaseFC (Alpha Innotech).

Estimation of protein:

Protein was estimated by the method described by Lowry's method (Lowry et al., 1951). This method involves the pre-treatment of protein with alkaline copper sulphate in the presence of tartrate followed by addition of Folin's phenol reagent (a mixture of phosphomolybdic-tungstic acids), which is reduced by the chelated protein. This results in the production of a characteristic blue colour that is measured spectrophotometrically at 660nm.

Reagents:

1. Lowry's reagent
2. Folin's reagent (diluted 1:1 with deionized water).

Assay: Samples were diluted 1:10 and added in volumes of 25 and 50µl. 2.5 ml of freshly prepared Lowry's reagent was added and the total volume was made up to 3 ml with deionized water. After incubating at room temperature for 10 minutes, 0.25 ml of Folin's phenol reagent was added. The blue colour produced was measured

spectrophotometrically at 660nm after incubating at room temperature for a further 30 minutes.

IV. Estimations in serum

Collection of blood from animals:

Blood (2ml) was collected by cardiac puncture after cervical dislocation in anesthetized animals that were sacrificed for removal of kidneys. Blood samples were allowed to clot for 30 minutes at room temperature and then centrifuged to separate out the serum. The serum samples were stored in airtight containers at –20°C for further analysis.

Blood (0.1ml) was collected by tail end sectioning in anesthetized animals for studies, which required repeated sampling.

Estimation of blood urea:

Blood urea levels were estimated using Infinity™ Urea single liquid stable reagent (Thermo Fisher Scientific, Inc.).

Estimation of serum creatinine:

The creatinine assay was based on the Jaffe's reaction in a protein free filtrate obtained from serum (Brod and Sirota, 1948). As the volumes of blood obtained from animals were small, the volumes in the above assay were modified to enable estimation in a ELISA plate reader. Proteins were precipitated by adding 60µl of deionized water, 20µl of 10% sodium tungstate and 40µl of 2/3N sulphuric acid to 40µl of serum. A protein-free filtrate was obtained as a supernatant by centrifugation of the above-mentioned

mixture at 3500 rpm for 5 minutes. 40 µl of working reagent (1:1 mixture of 0.004M picric acid and 0.75N NaOH) was added to 60 µl of protein free filtrate in a microtitre plate. The intensity of colour generated by the reaction was measured, using a Bio-Rad ELISA plate reader at a wavelength of 490nm. Serum creatinine was calculated on comparison with a 1mg% standard using the formula:

V. Serum creatinine (mg/dl) = Absorbance of test/ Absorbance of standard x 4

The precision of the above assay was established by plotting a standard graph and the accuracy was evaluated by using quality control samples obtained from the external QC program, Department of Clinical Biochemistry CMC Vellore.

VI. Statistical analysis

Data obtained from treated animals were compared with those from control animals. For the zymogrmas and Western blots, the bands from control animals were normalized with reference to the control. Statistical significance was calculated both within groups and between groups by perfoming a one way analysis of variance (ANOVA) with Bonferroni's as a posthoc test, using SPSS, version 11.0.A p value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Renal homogenates from rats showed pro-MMP-9 (92kD), pro-MMP-2 (72kd) and MMP-2 (65kd) activity.

1. Studies on proMMP-9

1.1. Effect of varying doses of indomethacin on pro-MMP-9 activity in rat kidney, 24 hours after the dose of the drug

Preliminary zymogram studies showed an initial increase in pro-MMP-9 activity, followed by a decrease in activity with increasing doses of indomethacin. Maximal activity was seen at a dose of 20mg/kg (Figures 1A and 1B).

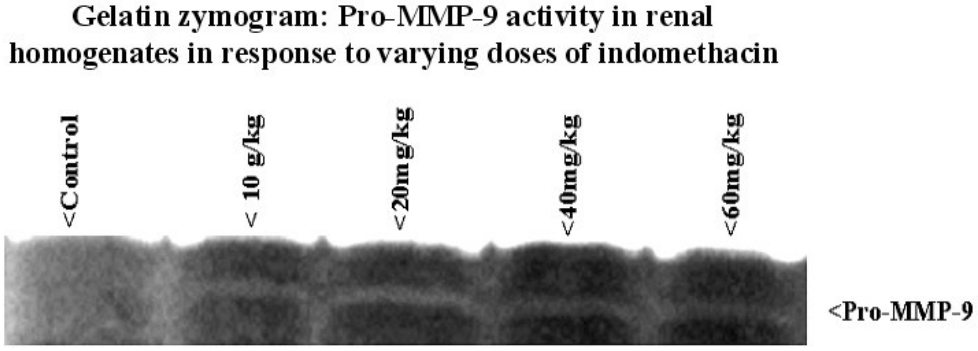


Figure 1A: Gelatin zymogram showing dose-dependent changes in proMMP-9 activity in response to indomethacin. Animals were treated with various doses of indomethacin and sacrificed 24 hours later. This is a representative zymogram showing MMP-9 activity (white bands), as assessed by gelatin zymography, using a 10% poly-acrylamide gel, loading 100 µg renal homogenate protein per lane. The samples were obtained from control (0mg/kg indomethacin) and indomethacin-treated (10mg/kg to 60mg/kg) animals.

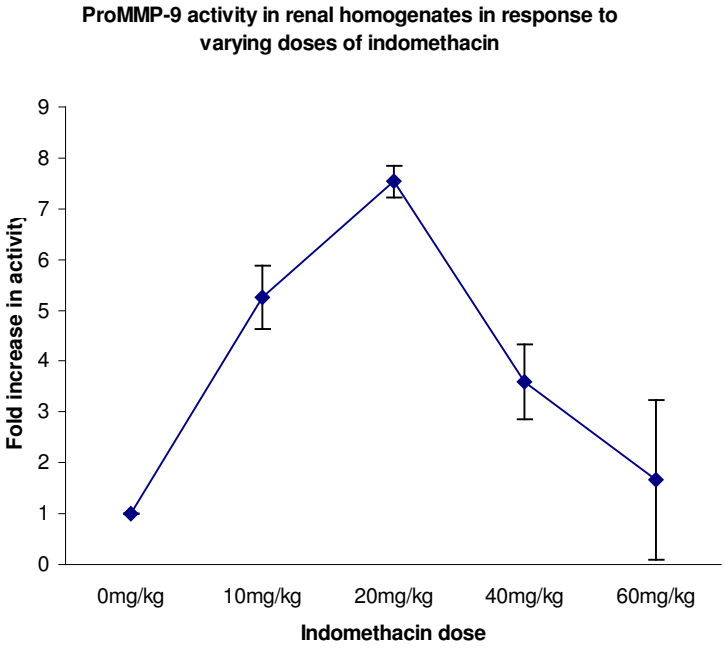


Figure 1B: Dose-dependent changes in pro-MMP-9 activity in response to indomethacin. Data are means of activity of pro-MMP-9 (± standard error of mean (SEM), obtained by image analysis, with the activity of the controls set at one.

1.2. Effect of varying doses of indomethacin on pro-MMP-9 protein levels in rat kidney, 24 hours after the dose of the drug

Western blot studies done with renal homogenates from animals given various doses of indomethacin showed that the drug did not produce any significant effects on levels of pro-MMP-9 protein (Figure 2A and 2B).

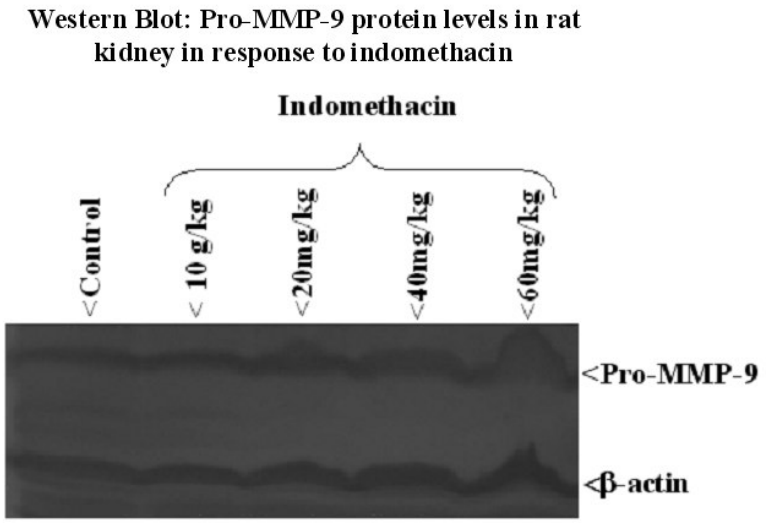


Figure 2A: Western blot showing effects of various doses of indomethacin on protein expression of proMMP-9 in renal homogenates, 24 hours after the drug. This is a representative blot, using a 10% poly-acrylamide gel, with 25 µg of protein loaded per lane. β-actin was used as a protein loading control.

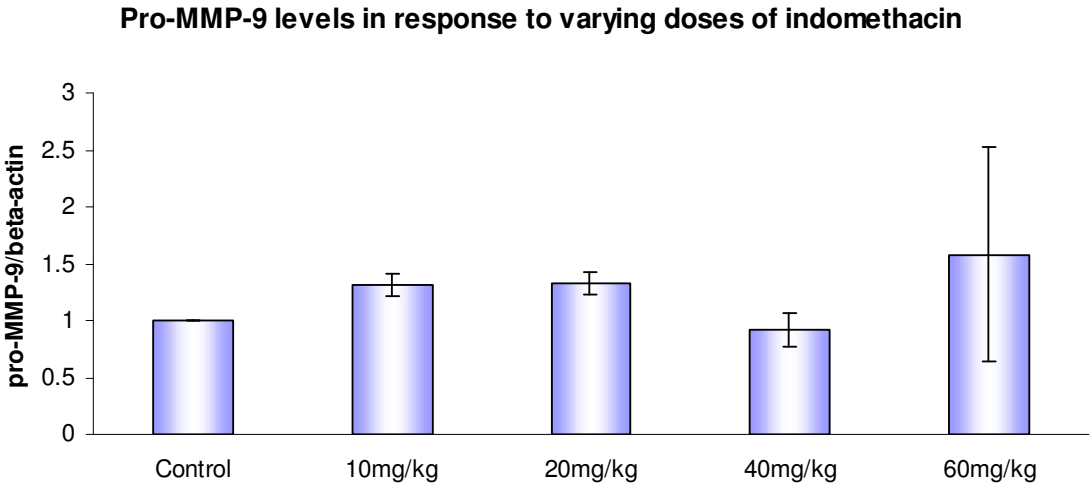


Figure 2B: Effects of various doses of indomethacin on protein expression of pro-MMP-9 in renal homogenates, 24 hours after the drug. Preliminary data shown here represent mean expression levels of pro-MMP-9 protein (± standard error of mean (SEM), n=2) obtained by image analysis of Western blots, with the activity of the controls set at one.

1.3. Effect of zinc pretreatment on indomethacin-induced effects on pro-MMP-9 in rat kidney, 24 hours after administration of indomethacin:

Indomethacin (at 20 mg/kg) was found to significantly increase pro-MMP-9 activity in the renal homogenates, as compared to the control groups (p value=0.012). Pretreatment of the animals with zinc did not produce any significant effect on the drug-induced increase in pro-MMP-9 activity (Figure 3). The protein levels of pro-MMP-9 were also not affected by the zinc pretreatment, either alone or with indomethacin (Figures 3A and 3B and Figures 4A and 4B).

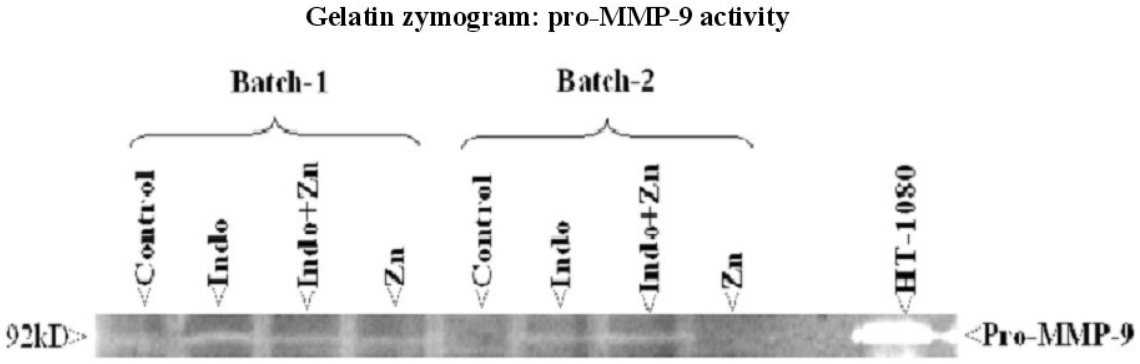


Figure 3A: Pro-MMP-9 (92kD) activity in renal homogenates, 24 hours after administration of 20mg/kg indomethacin, as assessed by gelatin zymography using a 10% poly-acrylamide gel, loading 100 µg protein per lane. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. HT-1080 cell line-conditioned media serves as a positive control for pro-MMP-9.

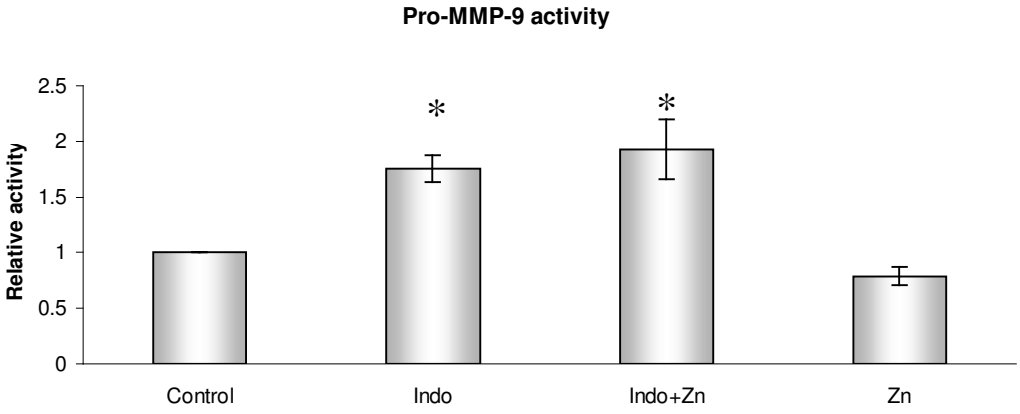


Figure 3B: Pro-MMP-9 (92kD) activity in renal homogenates. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. Data are means of activity of pro-MMP-9 (± SEM), obtained by image analysis of zymograms, with the activity of the controls set at one. * indicates $p<0.05$ as compared to control group; n=6.

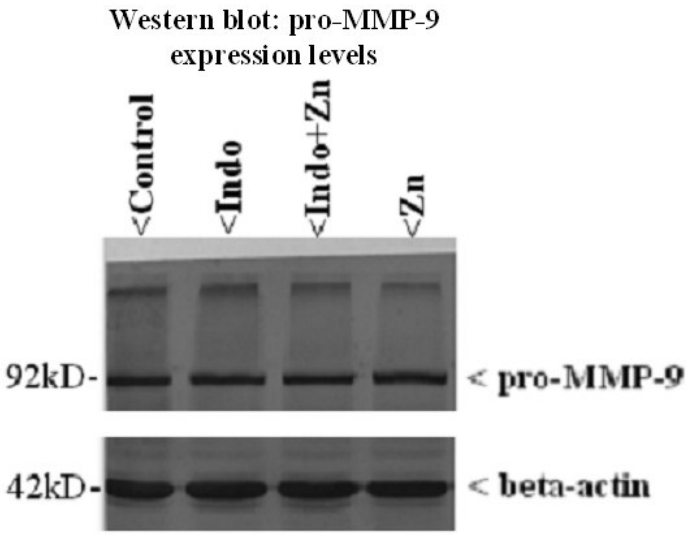


Figure 4A: Relative concentrations of pro-MMP-9 in renal homogenates, 24 hours after indomethacin (20mg/kg) administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 μg protein per lane. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups (n=5 in each group). This shows a representative blot with pro-MMP-9 (92kD) bands. β-actin bands show equal protein loading in all lanes.

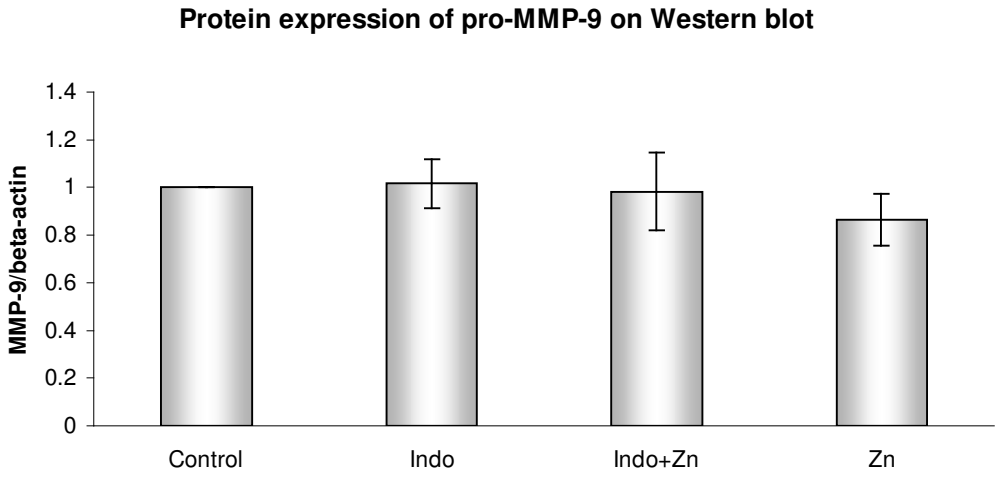


Figure 4B: Relative concentrations of pro-MMP-9 in renal homogenates, 24 hours after indomethacin (20mg/kg) administration, as assessed by Western blot. Data represents means of pro-MMP-9 protein levels (± SEM), obtained by image analysis of Western blots, with the concentration of the controls set at one.

2. Studies on pro-MMP-2

2.1. Effect of varying doses of indomethacin on pro-MMP-2 activity in rat kidney, 24 hours after the dose of the drug

Preliminary zymograms showed an initial rise in pro-MMP-2 activity, followed by a drop in activity with increasing doses of indomethacin. The maximal activity was noted at a dose of 10mg/kg at 24 hours (Figures 5A and 5B).

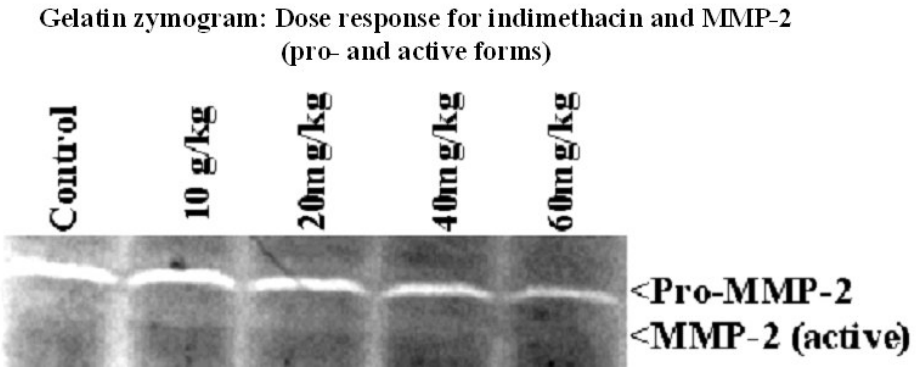


Figure 5A: Gelatin zymogram showing dose-dependent changes in pro-MMP-2 activity in response to indomethacin. Animals were treated with various doses of indomethacin and sacrificed 24 hours later. This is a representative zymogram showing MMP-2 activity (white bands), as assessed by gelatin zymography, using a 10% poly-acrylamide gel, loading 100µg renal homogenate protein per lane. The homogenates were obtained from control (0mg/kg indomethacin) and indomethacin-treated (10mg/kg to 60mg/kg) animals.

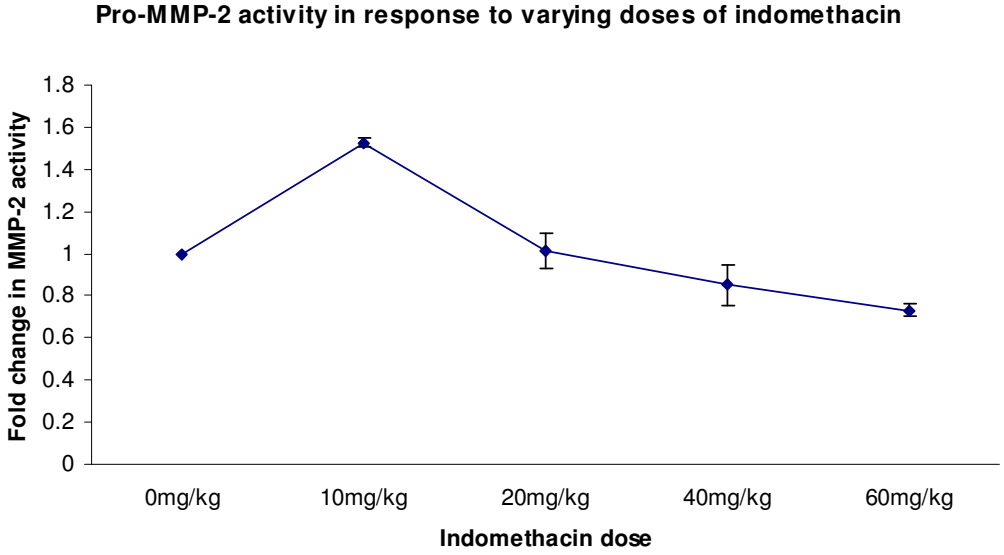


Figure 5B: ProMMP-2 activity in renal homogenates in response to varying doses of indomethacin. Data are means of pro-MMP-2 activity (±SEM), n=2, obtained by image analysis of zymograms, with the activity of the controls set at one.

2.2. Effect of varying doses of indomethacin on pro-MMP-2 protein levels in rat kidney, 24 hours after the dose of the drug

Western blots done to assess changes in pro-MMP-2 protein levels in response to increasing doses of indomethacin showed that there were no significant changes in the levels of protein with the different doses (Figure 6A and 6B).

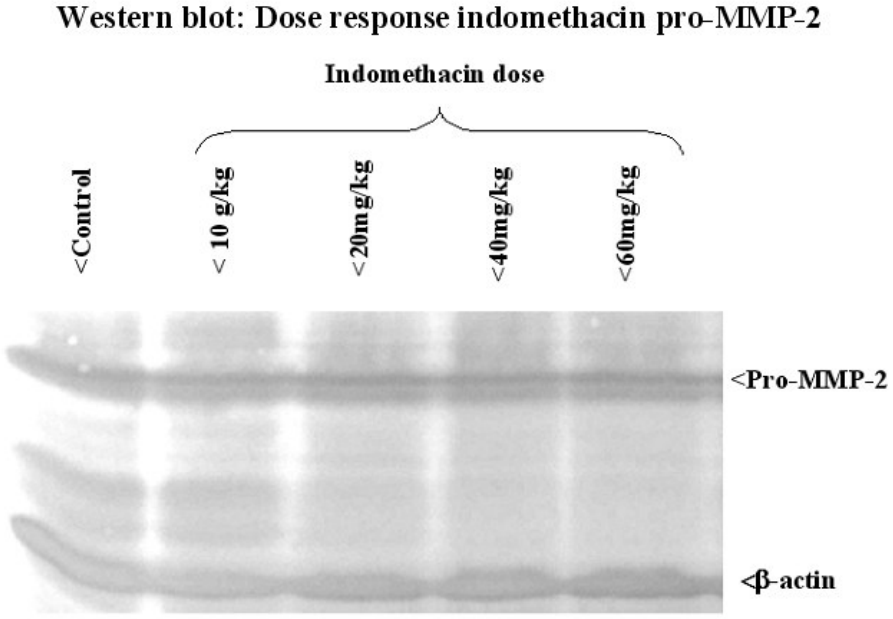


Figure 6A: Western blot showing effects of various doses of indomethacin on protein expression of pro-MMP-2 in renal homogenates, 24 hours after dosing. This is a representative blot, using a 10% poly-acrylamide gel, with 25 µg of protein loaded per lane. β-actin was used as a protein loading control.

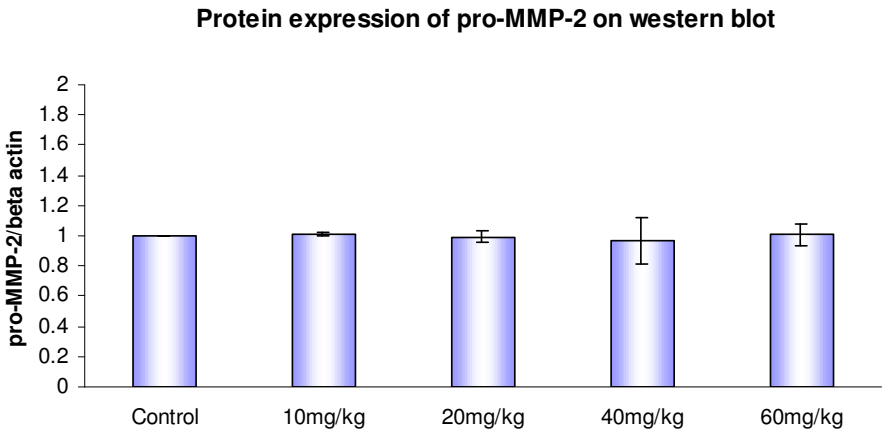


Figure 6B: Pro-MMP-2 activity in renal levels in response to varying doses of indomethacin. Data are means of pro-MMP-2 levels (±SEM), n=2, obtained by image analysis of western blots, with the activity of the controls set at one.

2.3. Effect of zinc pretreatment on indomethacin-induced effects on pro-MMP-2 in rat kidney, 24 hours after administration of indomethacin

Pretreatment with zinc did not result in any significant changes in indomethacin-induced changes in pro-MMP-2 activity (Figures 7A and 7B) or protein levels (Figures 8A and 8B). Treatment with zinc alone did not affect either the activity or protein content of proMMP-2.

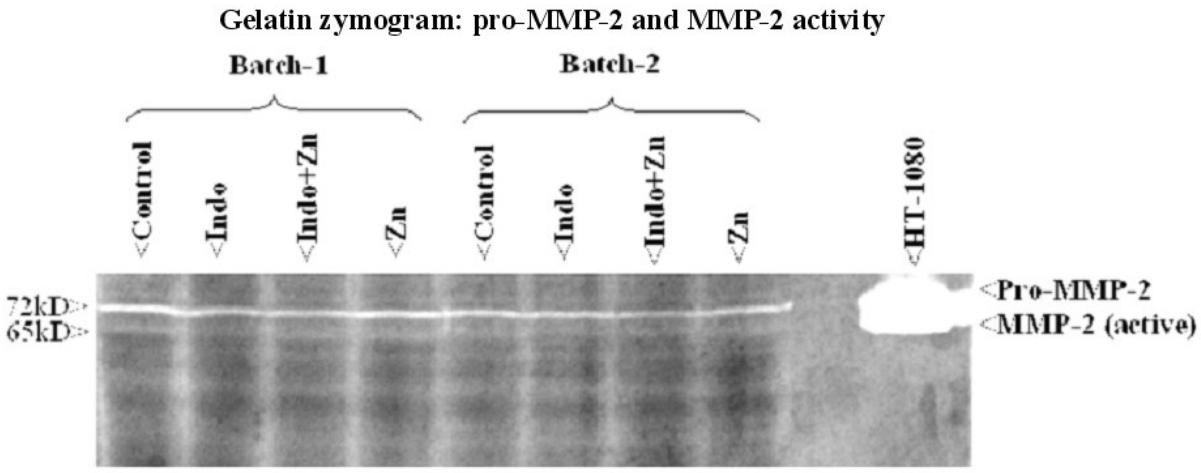


Figure 7A: Pro-MMP-2 (72kD) activity in renal homogenates, 24 hours after administration of 20mg/kg indomethacin, as assessed by gelatin zymography using a 10% poly-acrylamide gel, loading 100 µg protein per lane. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. HT-1080 cell line-conditioned media servesd as a positive control for pro-MMP-2.

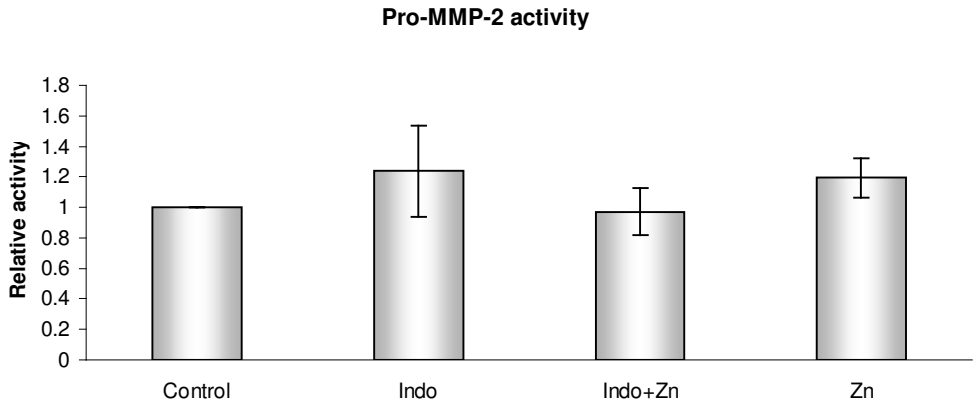


Figure 7B: Pro-MMP-2 (72kD) activity in renal homogenates. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. Data are means of activity of pro-MMP-2 (± SEM), obtained by image analysis of zymograms, with the activity of the controls set at one; n=6.

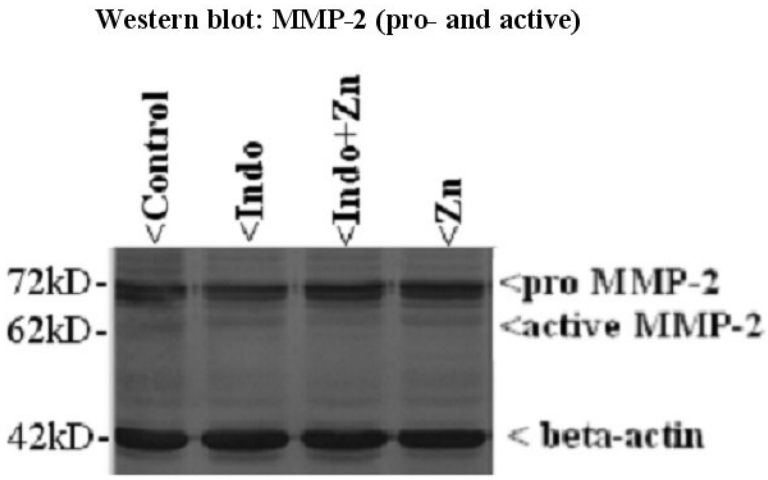


Figure 8A: Relative concentrations of pro-MMP-2 (72kD) in renal homogenates, 24 hours after indomethacin (20mg/kg) administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 µg protein per lane. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups (n=5 in each group). This shows a representative blot with pro-MMP-9 (92kD) bands. β-actin bands show equal protein loading in all lanes.

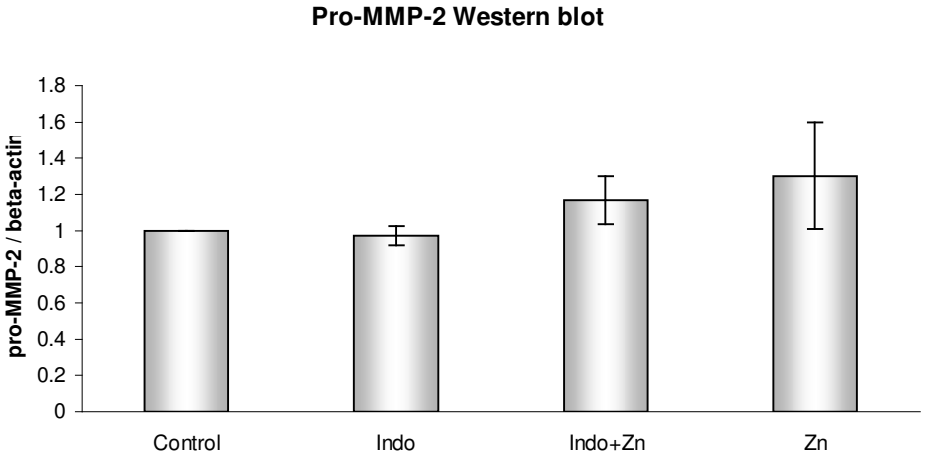


Figure 8B: Relative concentrations of pro-MMP-2 in renal homogenates, 24 hours after indomethacin administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 µg protein per lane. Data represents means of pro-MMP-2 protein levels (± SEM)), obtained by image analysis of Western blots with the concentration of the controls set at one.

3. Studies on MMP-2

3.1. Effect of varying doses of indomethacin on MMP-2 activity in rat kidney, 24 hours after the dose of the drug

Preliminary zymograms did not show any conclusive changes in the trends of MMP-2 activity (Figure 9A). The precision of densitometric measurements was reduced for MMP-2 as the intensity of the band was low. The bands for MMP-2 were not clearly visible in our preliminary western blots and are hence not reported.

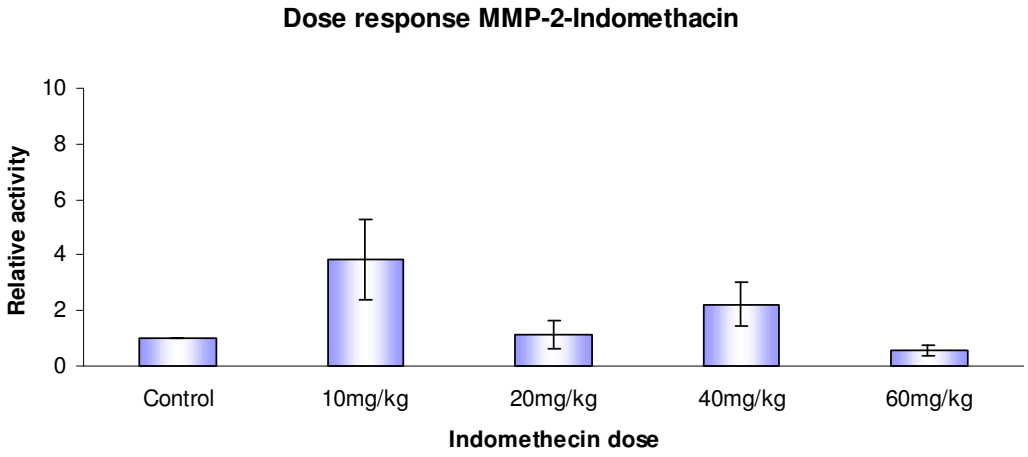


Figure 9A: MMP-2 activity in renal homogenates in response to varying doses of indomethacin. Data are means of MMP-2 activity (±SEM), n=2, obtained by image analysis of zymograms, with the activity of the controls set at one.

3.2. Effect of zinc pretreatment on indomethacin-induced effects on MMP-2 in rat kidney, 24 hours after administration of indomethacin

Analysis of data by ANOVA showed that there were overall significant differences between the groups. When applying the Bonferroni's correction as a post-hoc test, it was found that pretreatment with zinc in animals given indomethacin tended to increase MMP-2 activity, but the effect did not reach statistical significance ($p = 0.08$) (Figure 10A). Protein levels of MMP-2 were not affected by pre-treatment with zinc in any of the experimental groups (Figure 10B).

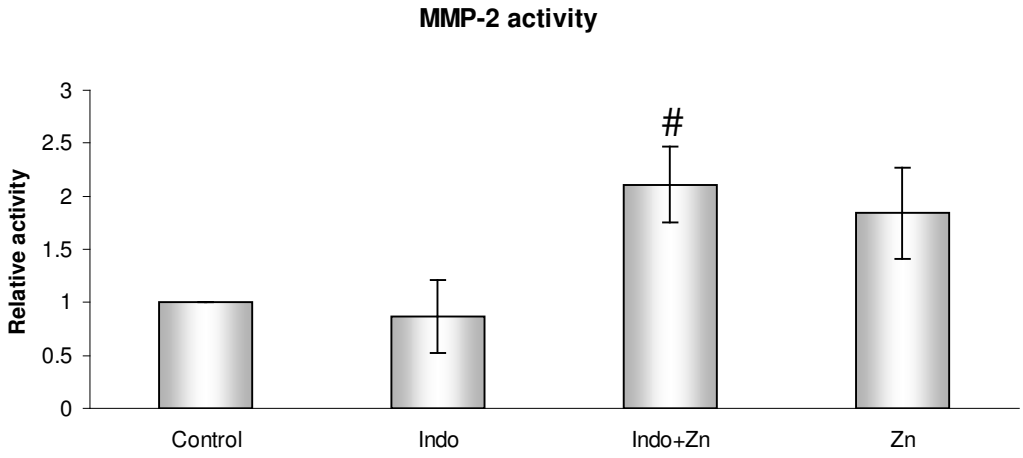


Figure 10A: MMP-2 (65kD) activity in renal homogenates. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. Data are means of activity of MMP-2 (± SEM), obtained by image analysis of zymograms, with the activity of the controls set at one; n=6.

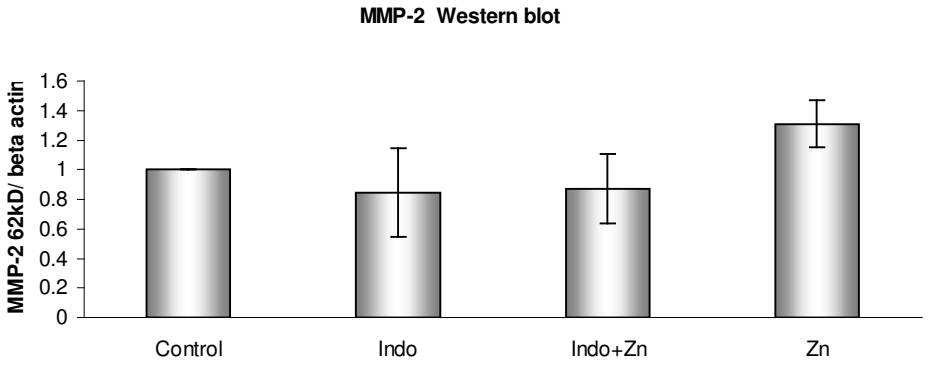


Figure 10B: Relative concentrations of MMP-2 in renal homogenates, 24 hours after indomethacin administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 µg protein per lane. Data represents means of MMP-2 protein levels (± SEM)), obtained by image analysis of Western blots with the concentration of the controls set at one.

4. Studies on TIMP-2

4.1. Effect of varying doses of indomethacin on levels of TIMP-2 in rat kidney, 24 hours after the dose of the drug

A preliminary study on the effects of varying doses of indomethacin on TIMP-2 expression levels, as assessed by Western blot, showed that the protein levels tended to decrease with increasing doses of indomethacin (Figures 11A and 11B).

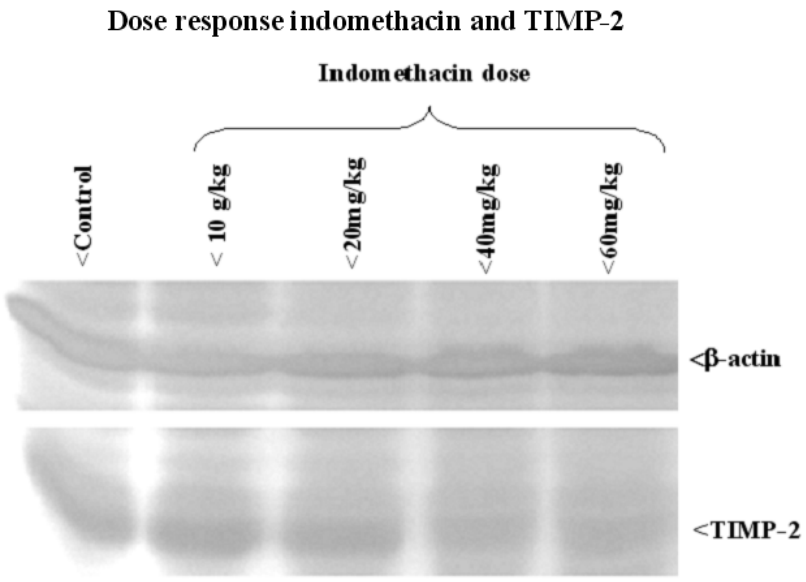


Figure 11A: Western blot showing effects of various doses of indomethacin on protein expression of TIMP-2 in renal homogenates, 24 hours after the drug. This is a representative blot of using a 10% poly-acrylamide gel, with 25 µg of protein loaded per lane. β-actin was used as a protein loading control.

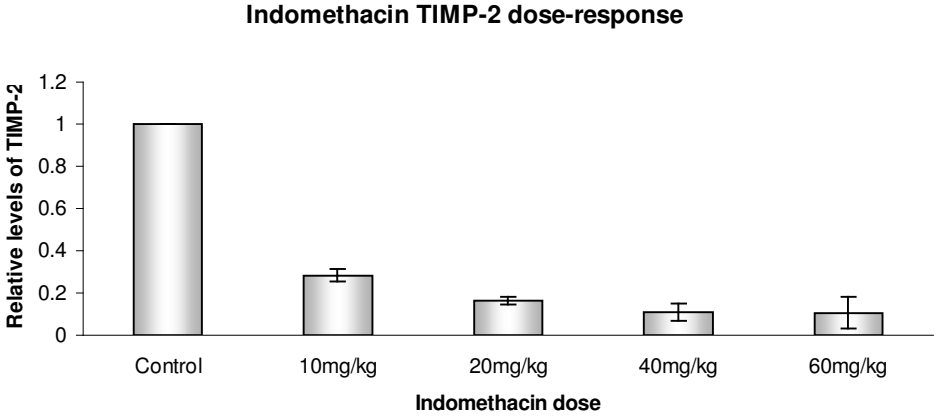


Figure 11B: Relative concentrations of TIMP-2 in renal homogenates, 24 hours after indomethacin administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 µg protein per lane. Data represents means of TIMP-2 protein levels ± standard error of mean (SEM)), obtained by image analysis of Western blots with the concentration of the controls set at one. n = 2

4.2. Effect of zinc pretreatment on indomethacin-induced effects on TIMP-2 in rat kidney, 24 hours after administration of indomethacin

Pretreatment with zinc did not affect indomethacin-induced changes in TIMP-2 (Figure 12A and 12B).

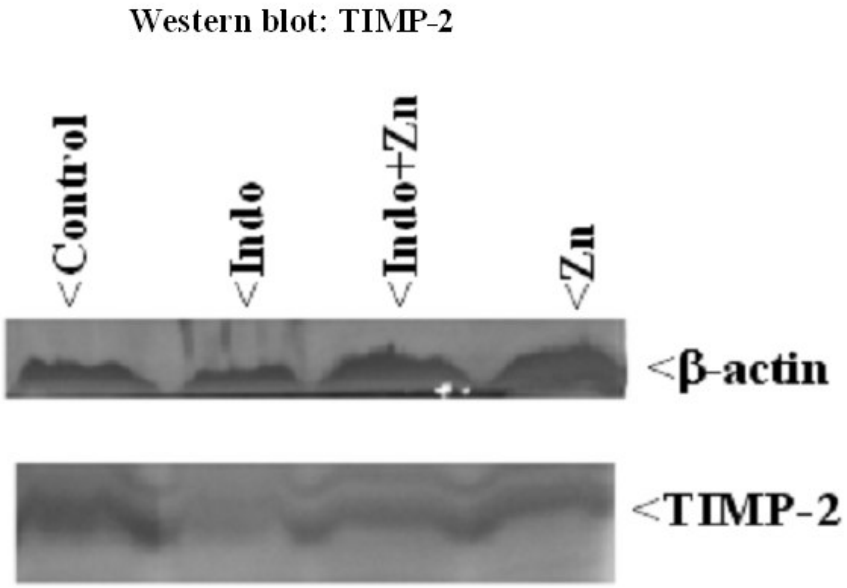


Figure 12A: Relative concentrations of TIMP-2 in renal homogenates, 24 hours after indomethacin (20mg/kg) administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 μg protein per lane. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups (n=6 in each group). This shows a representative blot with TIMP-2 bands. β-actin bands show equal protein loading in all lanes.

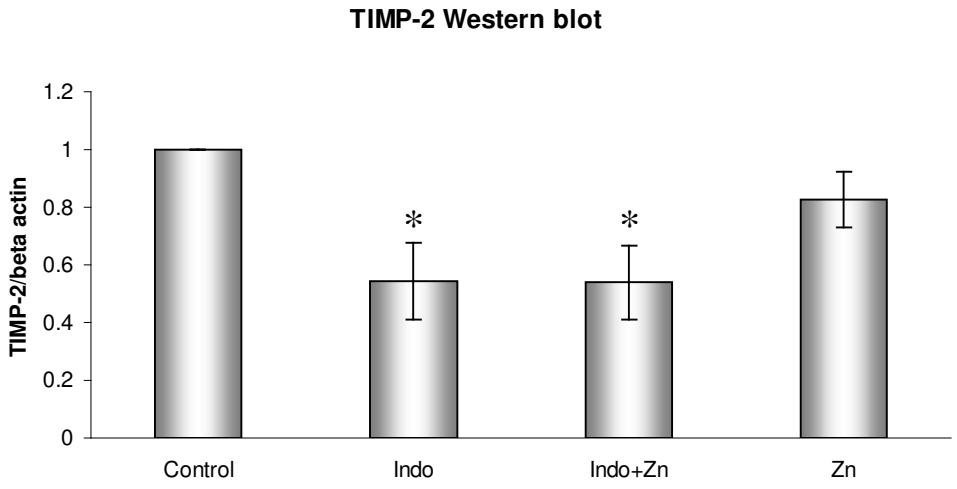


Figure 12B: Relative concentrations of TIMP-2 in renal homogenates, 24 hours after indomethacin administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 µg protein per lane. Data represent means of MMP-2 protein levels (± SEM)), obtained by image analysis of Western blots with the concentration of the controls set at one. * indicates $p<0.05$ as compared to control group.

5.Studies on TIMP-1

5.1. Effect of indomethacin (20mg/kg) on TIMP-1 levels in rat kidney, 24 hours after the dose of the drug

Indomethacin was found to significantly reduce TIMP-1 levels in the renal homogenate (Figure 13A).

5.2. Effect of zinc pretreatment on indomethacin-induced effects on TIMP-1 in rat kidney, 24 hours after administration of indomethacin:

Pretreatment with zinc showed a tendency to reverse the drug-induced decrease in TIMP-1 levels but the effect did not attain statistical significance (Figure 13B).

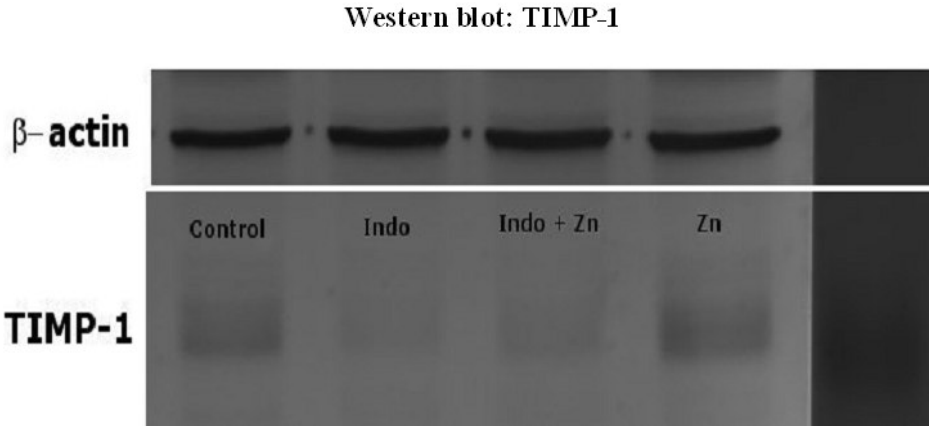


Figure 13A: Relative concentrations of TIMP-1 in renal homogenates, 24 hours after indomethacin (20mg/kg) administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 μg protein per lane. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups (n=4 in each group). This shows a representative blot with TIMP-1 bands. β-actin bands show equal protein loading in all lanes.

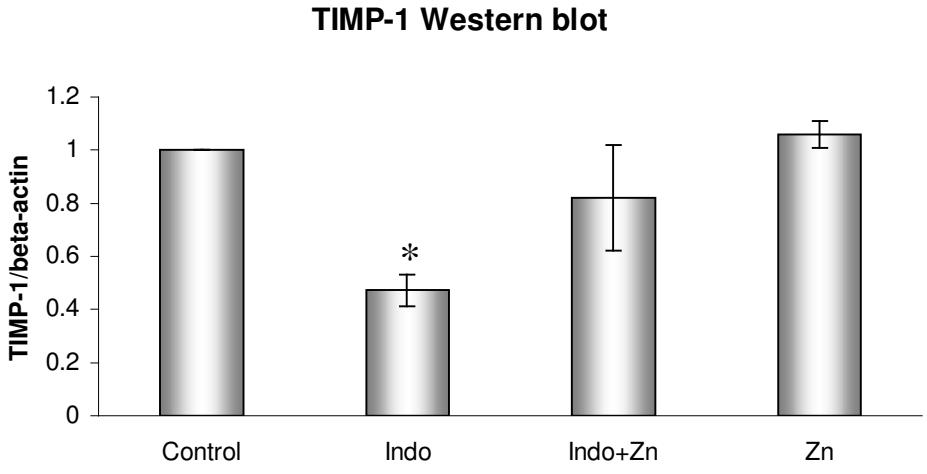


Figure 13B: Relative concentrations of TIMP-1 in renal homogenates, 24 hours after indomethacin administration, as assessed by Western blot using a 10% poly-acrylamide gel, loading 25 μg protein per lane. Data represent means of MMP-2 protein levels (± SEM), obtained by image analysis of Western blots with the concentration of the controls set at one. * indicates $p<0.05$ as compared to control group.

6. Studies on other gelatinases in the rat kidney

Gelatin zymograms showed the activity of other gelatinolytic enzymes in the rat kidney homogenates.

6.1. Effect of indomethacin on other gelatinases in the rat kidney 24 hours after the drug:

Treatment with indomethacin 20mg/kg did not significantly alter the activity of the other gelatinases in the rat kidney (Figures 14 and 15).

6.2. Effect of zinc pretreatment on indomethacin-induced effects on other gelatinases in the rat kidney, 24 hours after administration of indomethacin

Zinc pretreatment, either alone or before the indomethacin, did not produce any changes in the activity of these gelatinases (Figures 14 and 15)

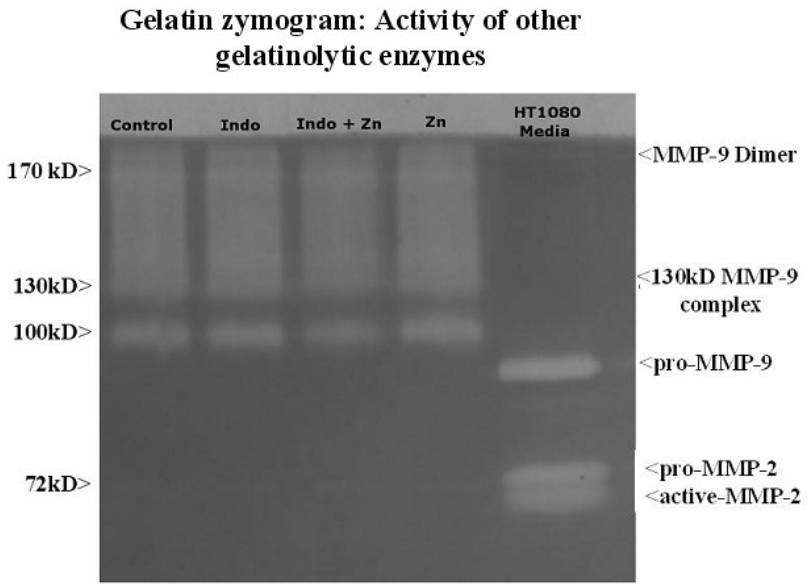


Figure 14A: Activity of other gelatinolytic enzymes 24 hours after the dose of indomethacin, as assessed by gelatin zymography on a 6% poly-acrylamide gel, loading 5 µg renal homogenate protein per lane, obtained from control, indomethacin treated (Indo), zincs pretreated (Indo + Zn)

and zinc (Zn) only groups. This is a representative zymogram (n=5) (A) showing white bands of molecular weights ~170kD, ~130kD and ~100kD with gelatinase activity. HT-1080 cell line media served as a positive control for the pro-MMP-9, pro-MMP-2 and MMP-2 activity.

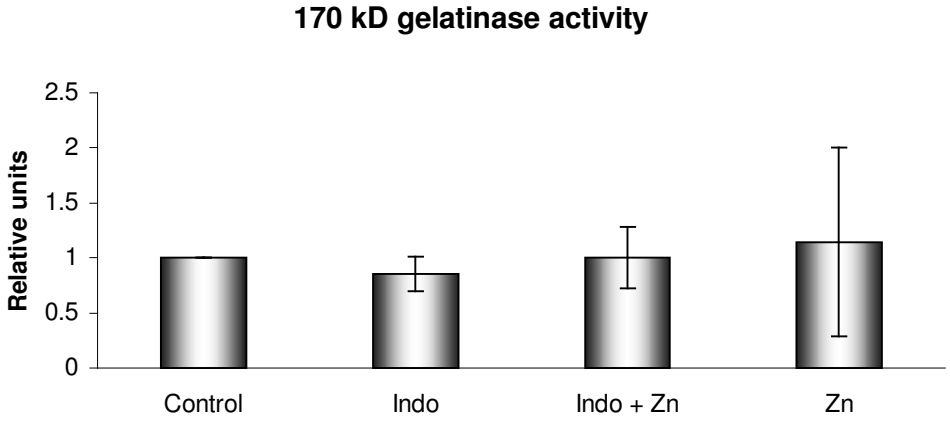


Figure 14B: 170 KD gelatinase activity in renal homogenates. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. Data are means of activity (± SEM), obtained by image analysis of zymograms, with the activity of the controls set at one; n=6.

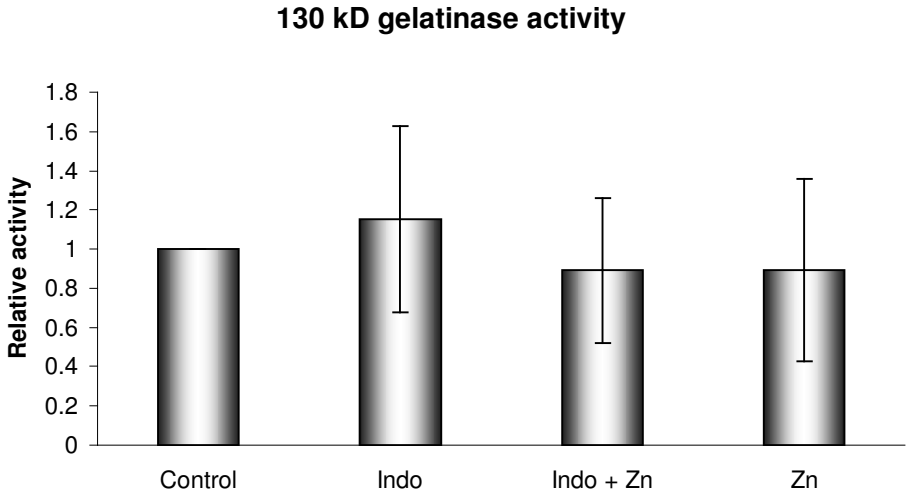


Figure 15A: 130 KD gelatinase activity in renal homogenates. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. Data are means of activity (± SEM), obtained by image analysis of zymograms, with the activity of the controls set at one; n=6.

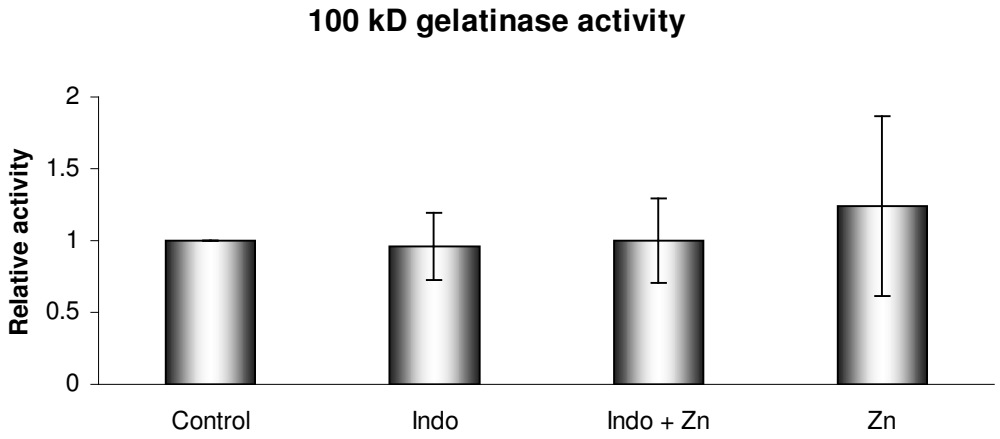


Figure 15B: 100 KD gelatinase activity in renal homogenates. The samples were from animals in the control, indomethacin-treated (Indo), zinc-pretreatment plus indomethacin (Indo + Zn) and zinc (Zn) only groups. Data are means of activity (± SEM), obtained by image analysis of zymograms, with the activity of the controls set at one; n=6.

7. Studies on assessment of renal function in the experimental animals

7.1. Effects of indomethacin and zinc pretreatment on serum creatinine and urea levels in rats

7.1.1. Effects of indomethacin on blood urea and serum creatinine levels 24 hours after the drug

The levels of blood urea and serum creatinine were found to be significantly higher in the indomethacin-treated rats as compared with levels in control animals (Figures 16A and 16B).

7.1.2. Effects of zinc pretreatment on indomethacin-induced changes in blood urea and serum creatinine levels 24 hours after the drug

Pre-treatment with zinc did not significantly alter indomethacin-induced increases in blood urea and serum creatinine 24 hours after the drug (Figures 16A and 16B).

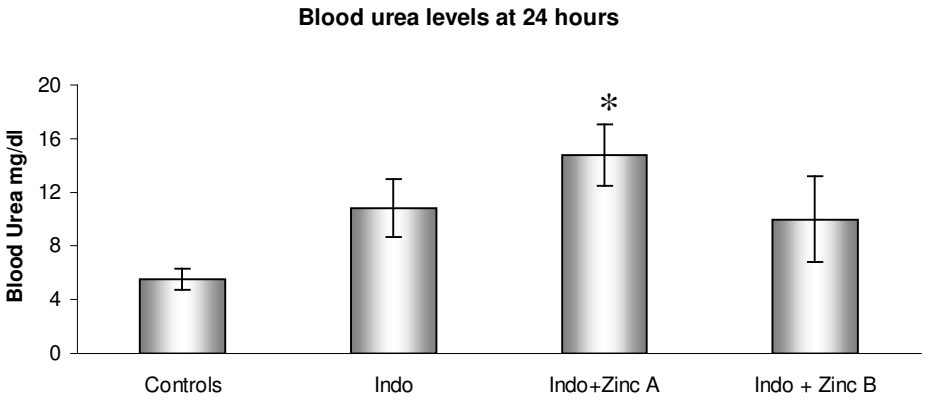


Figure 16A: Effects of indomethacin (20mg/kg body weight) on blood urea levels in rats sacrificed 24hours after the dose of drug. Data are represented as means (± SEM); n=5 for control; n=3 for other groups. * indicates $p<0.05$ as compared to control groups.

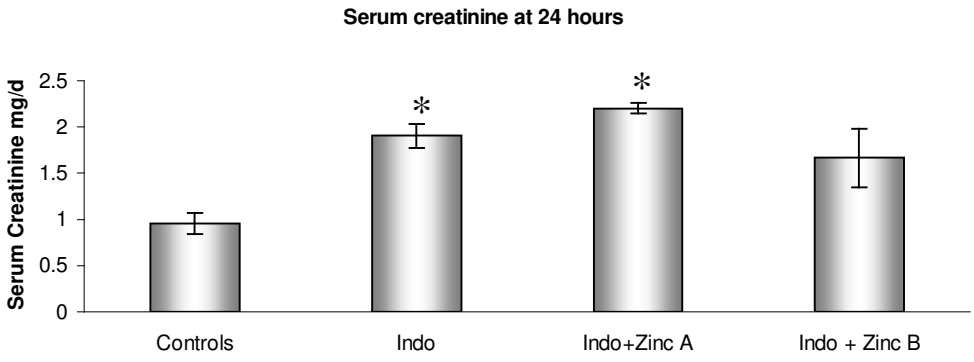


Figure 16B: Effects of indomethacin (20mg/kg body weight) on serum creatinine levels in rats sacrificed 24hours after the dose of drug. Data are represented as means (±SEM); n=5 for control; n=3 for other groups. * indicates $p<0.05$ as compared to control groups.

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7.1.3. Study on creatinine levels over time in animals treated with indomethacin, with and without pretreatment with zinc

Creatinine levels were increased to a significant extent in indomethacin-treated rats, from 12 hours (after the dose of indomethacin) onwards and remained at this level up to 72 hours, after which it began to fall, reaching close to baseline values by 120 hours. Animals pretreated with zinc showed similar trends but the levels began to fall earlier, i.e. soon after 24 hours. Treatment with zinc alone produced effects similar to Indo+ Zn on serum creatinine (Figure 17).

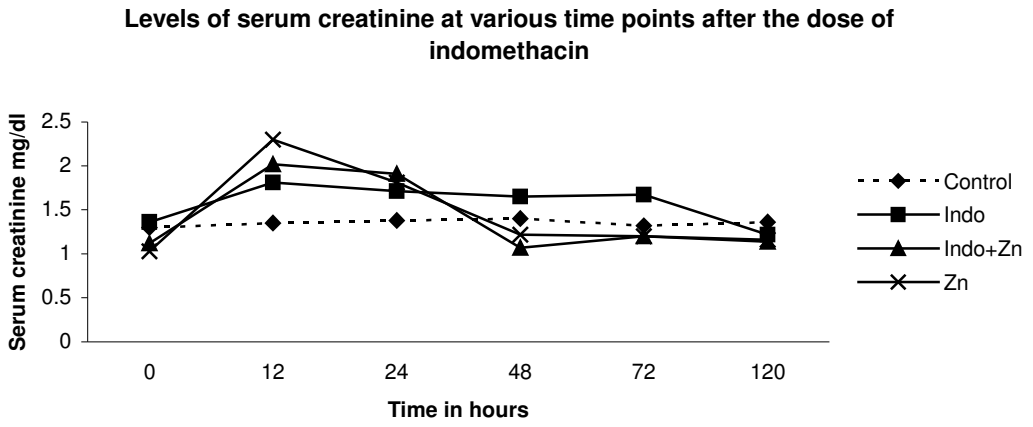

Figure 17: A preliminary (n=1) 5-day time course study of serum creatinine levels in control, indomethacin treated (Indo), zinc pretreated (Indo+Zn) and zinc (Zn) only groups.

DISCUSSION

This study attempted to determine whether matrix metalloproteinases-2 and -9 and their inhibitors, TIMP-1 and TIMP-2, are involved in the pathogenesis of indomethacin-induced renal dysfunction. We have shown that indomethacin, at 20mg/kg, increases pro-MMP-9 activity and decreases the levels of TIMP-1 and TIMP-2 in rat kidney. In addition, it is seen that these indomethacin-induced effects are not affected by prior administration of zinc. We also show that zinc does not affect indomethacin-induced changes in renal function, but it appears to tend to facilitate recovery from these changes.

Oxidative stress has been proposed as a critical initiator of indomethacin-induced renal damage (Basivireddy et al., 2004). The sources of oxidants resulting in such stress may be both in the intracellular and extracellular compartments. Changes in the balance of activities of pro- and antioxidant enzymes, a fall in glutathione levels and increased production of ROS by mitochondrial uncoupling are the major intracellular sources that contribute to oxidative stress (Somasundaram et al., 1997, Nieto et al., 2002, Villegas et al., 2002). Neutrophil infiltration and activation and reactive drug metabolites are the major causes for such a stress in an extracellular environment (Basivireddy et al., 2004, Lee et al., 1992, Asako et al., 1992). The reported effects of indomethacin-induced oxidative stress on the ultra-structure of renal tubular cells include mitochondrial swelling, intracytoplasmic vacuolation and inclusion bodies, all of which are pathological phenotypes resembling acute tubular injury (Basivireddy et al., 2004). Oxidative stress, also induced in other forms of acute renal injury, is a known modulator of extra-cellular matrix remodeling in the kidney (Sachse and Wolf, 2007). Thus, the drug-induced effects

seen in the kidney suggest the possibility that extra-cellular matrix remodelling may be an important factor in both the pathogenesis and recovery of indomethacin-induced renal damage. This prompted us to look at the effect of indomethacin on matrix metalloproteinases and their inhibitors TIMPs.

The preliminary dose response studies done showed an initial rise in the activity of pro-MMP-9, which was followed by a fall in activity. The peak activity was seen at a dose of 20mg/kg (Figure 1). Previous studies done in our laboratory have shown significant oxidative stress occurring in the kidney at this dose of indomethacin (Basivireddy et al., 2004). Analysis for changes in the protein content of pro-MMP-9, by Western blot, did not show any significant changes with increasing doses of indomethacin (Figure 2). These findings suggest an enhancement of pro-MMP-9 enzyme activity in the rat kidney without changes in the levels protein. This is in response to a 20mg/kg dose of indomethacin 24 hours after the drug.

Moderate elevations in the levels of reactive oxygen species have been reported to increase the activity of pro-MMP-9 (Rajagopalan et al., 1996). The process of S-glutathiolation of pro-MMP-9 has been described as a regulator of this increase in activity (Okamoto et al., 2001). Indomethacin is known to induce the generation of ROS and increase tissue levels of oxidized glutathione (Basivireddy et al., 2004, Nieto et al., 2002). Exposure of thiol-containing proteins to such environments induces S-glutathiolation (Chen et al., 2007, Shackelford et al., 2005). S-glutathiolation, in addition

to being a rapid regulator of enzyme activity, is a cellular defense mechanism, which maintains the stability of thiol groups during oxidant exposure (Shackelford et al., 2005). Based on these reports, we hypothesize that S-glutathiolation of pro-MMP-9 may be the mechanism involved in the increase in MMP-9 activity observed in our study.

High levels of oxidative stress have also been shown to oxidize vital amino-acid residues and inhibit pro-MMP-9 enzyme activity (Rajagopalan et al., 1996). It is possible that doses of indomethacin higher than 20mg/kg may induce proportionately greater changes in the redox environment. This implies that the fall in pro-MMP-9 activity observed at higher doses of indomethacin in this study may be a result of higher levels of oxidative stress. However, this is speculative and needs to be confirmed by estimating parameters of oxidative stress with increasing doses of indomethacin.

No significant changes were observed in pro-MMP-2 or cleaved MMP-2 activities or levels of their proteins in response to a 20mg/kg dose of indomethacin, 24 hours after the drug the drug (Figure 6 and Figure 9). Pro-MMP-2 activity was found to peak at a dose of 10mg/kg (Figure 5). Pro-MMP-2 activity at an indomethacin dose of 20mg/kg decreases to levels similar to that in the control group and hence does not appear to be significant at 24 hours. These are, however, preliminary studies. Further studies with a lower dose of indomethacin (10mg/kg) are required to determine whether significant effects of the drug on pro-MMP-2 and oxidative stress do occur at this dose. It is possible that oxidative stress may be involved in modulating the activity of pro-MMP-2 also. This would need to be confirmed with further studies.

Preliminary studies showed a decrease in the expression levels of TIMP-2 with increasing doses of indomethacin. The decreases were marked at all the doses of indomethacin used (Figure 11). Studies assessing TIMP-1 levels showed similar result with 20mg/kg indomethacin at 24 hours (Figure 13). These studies, thus, show that indomethacin, at a dose of 20mg/kg, reduced both TIMP-2 and TIMP-1 levels in the kidney.

The studies that followed were done to assess the effects of 20mg/kg dose indomethacin at 24 hours, at which time point, significant levels of oxidative stress were previously observed in the kidney (Basivireddy et al., 2004). The results confirmed the findings in the dose-response study and showed a significant decrease in TIMP-2 activity in the kidney on administering indomethacin.

Levels of TIMP-2 fell significantly with indomethacin, starting with the lowest dose of 10mg/kg (Figure 11B). This did not correlate with our observations on MMP-9 activity, which have shown marked increases in activity at doses of 10, 20 and 40 mg/kg (Figure 1B). It thus appears that TIMP-2 does not appear to be the major regulator of MMP-9 activity. Other known regulators of activity of this enzyme activity include tissue factor pathway inhibitor-2 (TFPI-2), thrombospondin 1 and 2, alpha-2 macroglobulin and reversal-inducing cysteine rich protein with Kazal motifs (RECK) (Visse and Nagase, 2003). These regulators as well in response to oxidative stress may influence the activity of pro-MMP-9. Thus is necessary to establish the role of these other regulators in response to oxidative stress in the kidney in further studies.

TIMP-1 has been shown to be inactivated by reactive oxygen species like $ONOO^-$ at low concentrations (<100μM) and in a dose-dependent manner (Frears et al., 1996). Higher concentrations (500μM-5mM) of peroxynitrite cause protein fragmentation (Frears et al., 1996). Studies also suggest a role of $ONOO^-$ in inactivating and decreasing TIMP-2 levels (Chakraborti et al., 2004). Peroxynitrate generation in response to indomethacin toxicity has been reported in the intestine (Konaka et al., 1999) and kidney (De Angelis et al., 2004). On the basis of these reports, we hypothesize that indomethacin-induced increases in peroxynitrates may be involved in causing the changes we have observed in the levels of TIMPs in the kidney. This would need to be confirmed by measuring peroxynitrates in the kidneys of the experimental animals.

These findings give insights into extracellular matrix turnover in acute indomethacin-induced renal toxicity. The results suggest a renal environment with high levels of pro-matrix-metalloproteinase–9 activity and low levels of the inhibitory proteins, TIMP-2 and TIMP–1. Such a state at this time point after the drug would suggest that matrix breakdown predominates over biosynthesis. Degradation of the tight junction protein, zonula occludens-1 between the tubular cells and occludin in endothelial cells in the glomerulus, is linked to the increased activity of MMP-9 in the kidney. This has been reported to result in tubular dysfunction and increased vascular permeability (Catania et al., 2007).

Zinc is a well-known antioxidant. Its efficacy in conferring protection to the gastric mucosa by virtue of its antioxidant actions has been previously reported (Joseph et al.,

1999). Studies done in our laboratory on the effects of zinc on indomethacin-induced oxidative damage in both the kidney (unpublished data) and intestine (Basivireddy et al, 2002 and 2003) have also demonstrated its protective effects. In our current study, it was found that zinc did not alter indomethacin-induced changes in matrix-metalloproteinases or their inhibitors. This observation was contrary to expectations. It, thus, appears that zinc does not produce its protective effect by affecting the activities of MMP and TIMP in the kidney. Its cytoprotective effects are thus likely to be mediated through other mechanisms.

It is important to note, that zinc is not an antioxidant by itself (Powell, 2000). The protection offered by zinc is attributed to both acute and chronic downstream effects. Acute effects of zinc include stabilization of sulfhydryl groups and displacement of redox-active transition metals from macromolecules. Chronic effects of the metal are mediated by the induction of metallothioneins, which act as ultimate antioxidants (Powell, 2000). It is interesting to note that both these mechanisms are most effective intracellularly. This is further supported by the fact that the concentrations of zinc are higher in the intracellular compartment than in the extracellular environment (Onosaka and Cherian, 1982). Plasma levels of zinc are very low and are, in fact, tightly regulated (Wise, 1995). Post-transcriptional modifications play a major role in the regulation of MMPs and their inhibitors. These changes most often occur at an extracellular site and hence may not be influenced by intracellular antioxidants. Thus, it may be that zinc is probably more active as an intracellular antioxidant and has very little role in mediating changes occurring in the extracellular environment. This may account for our findings

that zinc did not affect indomethacin-induced effects on MMP and TIMP in our studies. It would be interesting to assess the role of other antioxidants, such as TEMPOL that are effective in the extracellular environment, in further studies.

In an attempt to further study the protective effect of zinc, we looked at markers of renal function, viz., serum creatinine and blood urea 24 hours after the dose of indomethacin (Figure: 16). We found that indomethacin at a dose of 20mg/kg significantly increased levels of both blood urea and serum creatinine. Pre-treatment with zinc did not significantly affect these drug-induced changes. We concluded that from these data that zinc does not directly affect the action of indomethacin on renal function. The rise in urea and creatinine are likely to be due to a drug-induced decrease in the GFR, an effect that has been reported in response to administration of NSAIDs (Morgan and Anderson, 2003). Since zinc confers antioxidant protection primarily within cells, we hypothesized that the zinc may hasten recovery of indomethacin-induced impairment of renal function. We did a preliminary time course study to ascertain if our hypothesis was correct (Figure: 17). The results of this study appear to indicate that it is so. However, this would need confirmation with more animals being studied.

Another intriguing finding we have observed is that that animals given zinc alone also demonstrated increases in serum creatinine. The cause for this effect is yet unknown and needs to be confirmed with more studies. If found to be consistent, it is a finding that will be of interest and will merit future studies.

- ***STUDY LIMITATIONS***

The limitations of this study is as follows:

1. The sample size in our preliminary studies is limited to two batches. In order to comment on the significance of the obtained trends we must have a minimum sample size of six batches for analysis.
2. We base many of our findings on the assumption that increasing doses of indomethacin cause a propotional increase in renal oxidative stress. A dose-response study on levels of oxidative stress in the kidney in response to increasing doses of indomethacin is a must to acertian this assumption.

The above-mentioned limitations are solely due to constraints of time. We are attempting to complete our study and plug these deficiencies in course of time.

CONCLUSION

Indomethacin, a prototypical NSAID increases pro-MMP-9 activity, decreases the expression levels of TIMP-1 and TIMP-2 and induces renal dysfunction at doses that induce significant oxidative stress in the kidney. The intracellular antioxidant zinc does not reverse these changes.

In conclusion, this study has enabled us to gain a better understanding of the role of the proteinase-antiproteinase balance in indomethacin-induced renal injury. It further extends our knowledge on the role of zinc as an agent to reduce NSAID-induced renal damage.

FUTURE PLANS

The future plans for this study is as follows:

1. We plan to establish the changes in MMP-binding of TIMP-1 and TIMP-2 in response to indomethacin induced oxidative stress by reverse zymography.
2. The role of zinc in hastening recovery in NSAID mediated renal damage must be evaluated.
3. We would like to evaluate the effects of extracellular antioxidants on the changes in MMP activity.
4. We intend to study role of nitrosative stress in indomethacin induced renal toxicity.
5. We intend to study histopathological changes associated with indomethacin toxicity and correlate this with changes in MMP activity in the kidney by in-situ-zymography.

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Appendix I

1. Raw and normalized data from preliminary study:

Zymogram: Dose response indomethacin vs. pro-MMP-9 (preliminary data); raw data					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	549	3231	4137	2373	1775
Batch 2	549	2544	4394	1571	53
All values are as obtained on densitometric quantification of zymograms and are expressed as arbitary units					

Zymogram: Dose response indomethacin vs. pro-MMP-9 (preliminary data); normalized data (as a ratio of the control value) shown in Figure 1B					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	1	5.885246	7.535519	4.322404	3.233151
Batch 2	1	4.63388	8.166742	2.861566	0.096539
All values are as obtained on densitometric quantification of zymograms and are expressed as ratios					

Zymogram: Dose response indomethacin vs. pro-MMP-2 (preliminary data); raw data					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	12971	19535	14221	12233	9876
Batch 2	28538[1]	20049	26459[1]	9824	9076
All values are as obtained on densitometric quantification of zymograms and are expressed as arbitary units [1] results from a different gel compared to each other for normalization					

Zymogram: Dose response indomethacin vs. pro-MMP-2 (preliminary data); normalized data (as a ratio of the control value) shown in Figure 5B					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	1	1.506052	1.096369	0.943104	0.761391
Batch 2	1	1.545679	0.92715	0.757382	0.699715
All values are as obtained on densitometric quantification of zymograms and are expressed as ratios					

Zymogram: Dose response indomethacin vs. MMP-2 activity (preliminary data); raw data					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	2931	8182	4434	8110	1989
Batch 2	5535	14232	4431	4909	1187
All values are as obtained on densitometric quantification of zymograms and are expressed as arbitary units					

Zymogram: Dose response indomethacin vs. MMP-2 activity (preliminary data); normalized data (as a ratio of the control value) shown in Figure 9A					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	1	2.791539	1.512794	2.766974	0.678608
Batch 2	1	4.855681	0.80054	1.674855	0.404981
All values are as obtained on densitometric quantification of zymograms and are expressed as ratios					

Western blot: Dose response indomethacin vs. pro-MMP-9 levels (preliminary data); normalized data as shown in Figure 2B					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	1	1.217296	1.328207	0.764003	0.640962
Batch 2	1	1.414008	1.29635	1.067972	2.521795
All values are as obtained on densitometric quantification of western blots and are expressed as ratios					

Western blot: Dose response indomethacin vs. pro-MMP-2 levels (preliminary data); normalized data as shown in figure 6B					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	1	1.002556	0.957317	0.81371	0.934626
Batch 2	1	1.02387	1.03092	1.122563	1.076759
All values are as obtained on densitometric quantification of western blots and are expressed as ratios					

Western blot: Dose response indomethacin vs. TIMP-2 levels (preliminary data); normalized data (as a ratio of the control value) shown in Figure 11B					
	0	**10mg/kg**	**20mg/kg**	**40mg/kg**	**60mg/kg**
Batch 1	1	0.311944	0.146998	0.066769	0.03337
Batch 2	1	0.254419	0.18353	0.151006	0.179641
All values are as obtained on densitometric quantification of western blots and are expressed as ratios					

2. Zymography and western blot data with 20mg/kg indomethacin dosing

Zymogram: Pro-MMP-9 activity; raw data				
	Control	**Indo**	**Indo+Zn**	**Zn**
• ***Batch 1***	• 20884	35190	41318	10592
Batch 2	13077	24310	35954	11849
Batch 3	14668	26006	12926	10003
Batch 4	7125	15903	15970	6553
Batch 5	10363	13812	15287	10826
Batch 6	4213	6928	9490	2832
All values are as obtained on densitometric quantification of zymograms and are expressed as arbitary units				

Zymogram: Pro-MMP-9 activity; normalized data (as a ratio of the control value) shown in Figure 3B				
	Control	**Indo**	**Indo+Zn**	**Zn**
• ***Batch 1***	• 1	1.685022	1.978452	0.507183
Batch 2	1	1.858989	2.749407	0.906095
Batch 3	1	1.772975	0.881238	0.681961
Batch 4	1	2.232	2.241404	0.919719
Batch 5	1	1.332819	1.475152	1.044678
Batch 6	1	1.644434	2.252552	0.672205
All values are as obtained on densitometric quantification of zymograms and are expressed as ratios				

Zymogram: Pro-MMP-2 activity; raw data				
	Control	**Indo**	**Indo+Zn**	**Zn**
• ***Batch 1***	54105	36436	84457	65808
Batch 2	30433	73047	40598	46242
Batch 3	28538	26459	19815	29043
Batch 4	24537	46600	17719	26547
Batch 5	20403	12862	15939	32173
Batch 6	24860	22484	18026	18562
All values are as obtained on densitometric quantification of zymograms and are expressed as arbitary units				

Zymogram: Pro-MMP-2 activity; normalized data (as a ratio of the control value) shown in Figure 7B				
	Control	**Indo**	**Indo+Zn**	**Zn**
• ***Batch 1***	1	0.673431	1.560983	1.216302
Batch 2	1	2.400256	1.334012	1.519469
Batch 3	1	0.92715	0.694337	1.017696
Batch 4	1	1.899173	0.722134	1.081917
Batch 5	1	0.630397	0.781209	1.576876
Batch 6	1	0.904425	0.725101	0.746661
All values are as obtained on densitometric quantification of zymograms and are expressed as ratios				

Zymogram: MMP-2 (active) activity; raw data

	Control	Indo	Indo+Zn	Zn
• ***Batch 1***	13113	3889	19309	21735
Batch 2	7220	2218	13510	12797
Batch 3	5487	3158	13233	11753
Batch 4	2137	5441	7938	7945
Batch 5	5535	4431	7104	5031
Batch 6	6829	4581	12929	5830

All values are as obtained on densitometric quantification of zymograms and are expressed as arbitary units

Zymogram: MMP-2 (active) activity; normalized data (as a ratio of the control value) shown in Figure 10A

	Control	Indo	Indo+Zn	Zn
• ***Batch 1***	1	0.296576	1.472508	1.657515
Batch 2	1	0.307202	1.871191	1.772438
Batch 3	1	0.575542	2.4117	2.141972
Batch 4	1	2.546093	3.714553	3.717829
Batch 5	1	0.800542	1.283469	0.908943
Batch 6	1	0.670816	1.893249	0.853712

All values are as obtained on densitometric quantification of zymograms and are expressed as ratios

Western blot: Pro-MMP-9/β-actin levels; normalized data (as a ratio of the control value) shown in Figure 4B				
	Control	**Indo**	**Indo+Zn**	**Zn**
• ***Batch 1***	1	1.116147741	1.244672557	1.188461538
Batch 2	1	0.922498154	0.952920412	0.968459945
Batch 3	1	1.071343639	1.397877984	0.818477554
Batch 4	1	0.673733172	0.861665014	0.820560229
Batch 5	1	1.296352523	0.455407645	0.527039106
All values are as obtained on normalization of densitometric quantification of western blot and are expressed as ratios				

Western blot: Pro-MMP-2/β-actin levels; normalized data (as a ratio of the control value) shown in Figure 7B				
	Control	**Indo**	**Indo+Zn**	**Zn**
• ***Batch 1***	1	0.879703489	1.215255865	1.07357022
Batch 2	1	1.09375	1.049744077	1.07664679
Batch 3	1	1.030915577	1.194960212	0.685751464
Batch 4	1	0.821023288	1.601812026	1.253395222
Batch 5	1	1.037340521	0.776130764	2.426864785

All values are as obtained on normalization of densitometric quantification of western blot and are expressed as ratios

Western blot: MMP-2/β-actin levels; normalized data (as a ratio of the control value) shown in Figure 10B

	Control	Indo	Indo+Zn	Zn
• ***Batch 1***	1	0.467120181	0.518717475	0.511801242
Batch 2	1	0.379464286	0.944021676	1.549586777
Batch 3	1	0.488505747	0.544871795	0.962264151
Batch 4	1	1.826659972	1.577759456	1.689008043
Batch 5	1	0.691460737	0.409087469	1.040571266

All values are as obtained on normalization of densitometric quantification of western blot and are expressed as ratios

Western blot: TIMP-2/β-actin levels; normalized data (as a ratio of the control value) shown in Figure 12B

	Control	Indo	Indo+Zn	Zn
• ***Batch 1***	1	0.797994	0.798992	1.014535
Batch 2	1	0.262911	0.434491	0.29353
Batch 3	1	0.937899	0.974542	0.997927

Batch 4	1	0.183528	0.057588	0.285932
Batch 5	1	0.388891	0.282928	0.679895
Batch 6	1	1.003787	0.965801	0.857938
All values are as obtained on normalization of densitometric quantification of western blot and are expressed as ratios				

Western blot: TIMP-1/β-actin levels; normalized data (as a ratio of the control value) shown in Figure 13B				
	Control	**Indo**	**Indo+Zn**	**Zn**
• ***Batch 1***	1	0.572944	1.18326	1.019159
Batch 2	1	0.302662	0.443319	1.112235
Batch 3	1	0.508587	0.507956	0.941008
Batch 4	1	0.506012	1.14883	1.167765
All values are as obtained on normalization of densitometric quantification of western blot and are expressed as ratios				

3. Data for estimation of blood urea and serum creatinine:

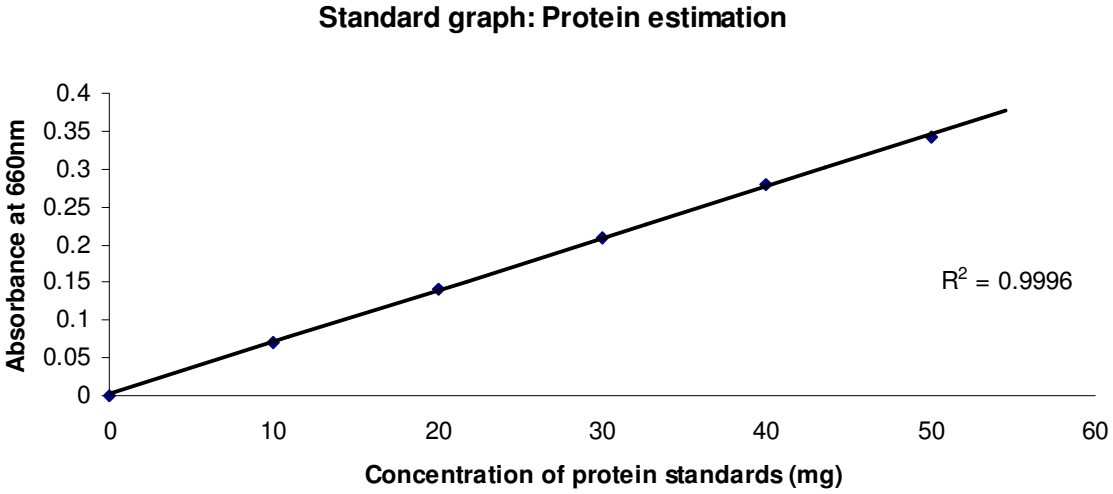
Blood urea levels in rats (mg%)				
	Control	**Indo**	**Indo+Zn A**	**Indo+Zn B**

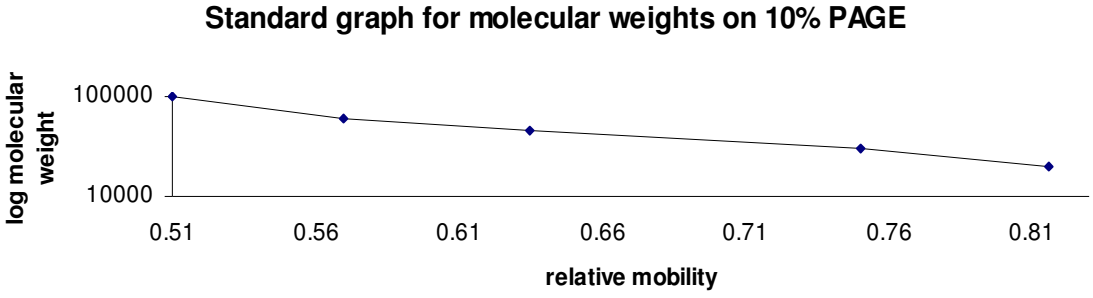
• ***Batch 1***	6.43	12.5	15	13.21
Batch 2	6.07	16.07	18.57	6.79
Batch 3	2.86	7.14	10.71	
Batch 4	4.64	7.5		
Batch 5	7.5			

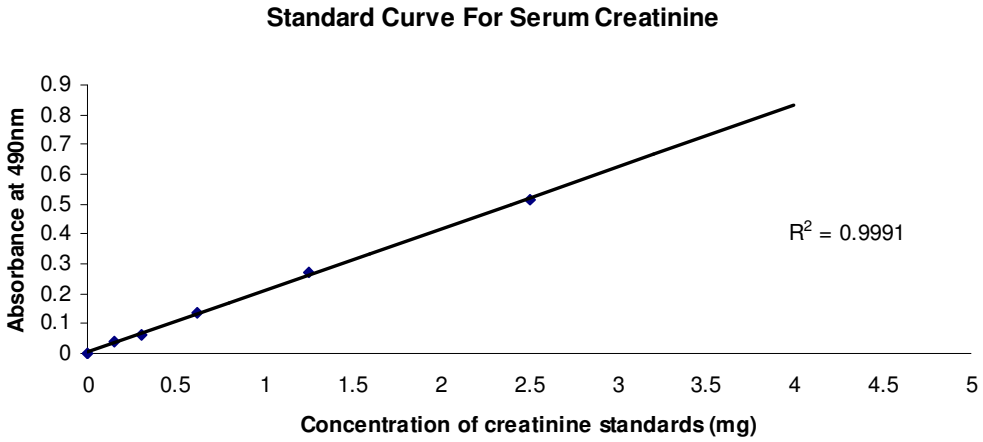
Serum creatinine levels in rats (mg%)				
	Control	**Indo**	**Indo+Zn A**	**Indo+Zn B**
• ***Batch 1***	1.1	1.7	2.3	1.7
Batch 2	0.81	1.7	2.3	2.2
Batch 3	1.2	2.25	2.1	1.1
Batch 4	0.72	1.97	2.1	

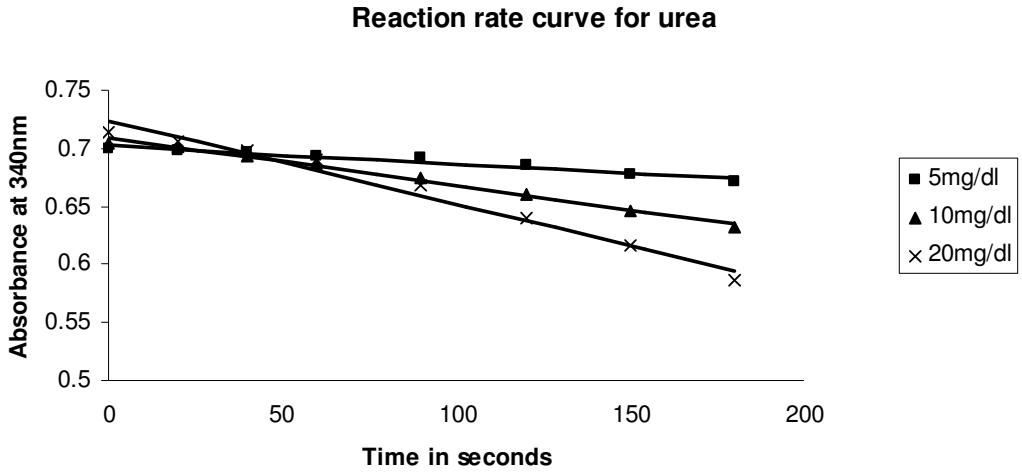
Appendix II

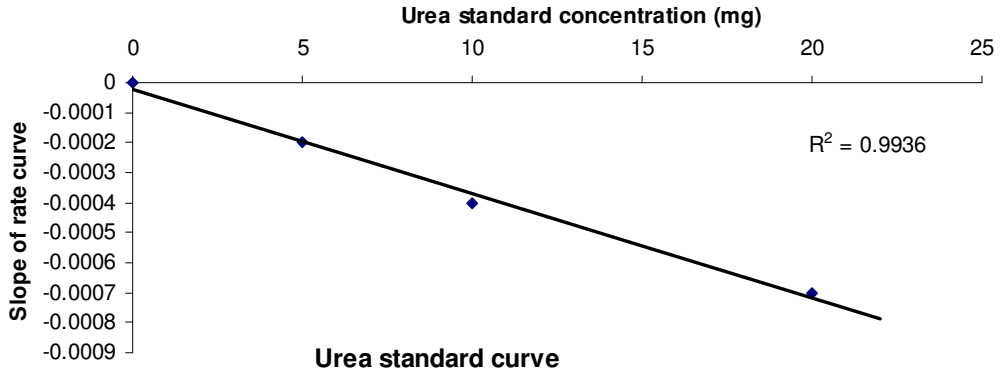
Standard curves






Standard Curve For Serum Creatinine
Absorbance at 490nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Concentration of creatinine standards (mg)
R2 = 0.9991


Reaction rate curve for urea
Absorbance at 340nm
0.75
0.7
0.65
0.6
0.55
0.5
0
50
100
150
200
Time in seconds
5mg/dl
10mg/dl
20mg/dl


Urea standard concentration (mg)
0
5
10
15
20
25
Slope of rate curve
0
-0.0001
-0.0002
-0.0003
-0.0004
-0.0005
-0.0006
-0.0007
-0.0008
-0.0009
R2 = 0.9936
Urea standard curve